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STUDIES ON THE BEHAVIOUR OF SOME MICROBIAL ASSOCIATIONS IN SOIL.

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D E D I C A T I O N

This work is dedicated to my mother and father. Without their sacrifices and encouragement, this thesis would not have been possible.

D E C L A R A T I O N

I declare that this thesis is my own composition, that the work of which it is a record has been carried out by myself and that it has not been submitted in any previous application for a higher degree.

This thesis describes results of research carried out in the Department of Microbiology, University of Edinburgh between October 1970 and October 1973.

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C O N T E N T S

S U M M A R Y.

(1) The effects of the physical, chemical and biological attributes of the soil environment on micro-organisms and soil microbial community dynamics are discussed.

(2) An investigation of the adequacy of available enumerative techniques for the study of soil microbial community dynamics is described. It was concluded that the Jones and Mollison (1948) agar film method was the most satisfactory and generally applicable means of enumeration. It was also concluded that cultural methods are only of value in studies of dynamics when they enumerate a single type of organism.

(3) A theoretical method for estimating the total number of actively-dividing non-mycelial micro-organisms in a soil sample is described and methods for assessing its applicability are discussed. Experiments were carried out with genetically labelled microbial populations in an attempt to verify the usefulness of this method. This model culture system proved inadequate for the testing of the method.

(4) A method for the quantitative assay of catabolically active micro-organisms in soil is described. This involved visual discrimination between cells on the basis of possession of active electron transport systems. Discrimination was made possible by cyto-

chemical demonstration of enzyme activity and was carried out in conjunction with Jones and Mollison enumeration.

(5) The nature of and relationship between microbial growth and activity in a model soil subjected to a point-stimulus was investigated. Growth and activity occurred in a cyclic manner. The period of the oscillations was approximately 40 hours and biomass production varied between 0 and 3.75 mg/g soil while CO_2 production varied between 0 and 5.2 μmoles/g soil. The implications of these findings are discussed.

(6) The concept of characterising a microbial community in terms of a 'fingerprint' made up of a frequency distribution in a parameter which can be split into appropriate class intervals is introduced. The concept is exemplified by the application of numerical and volumetric cell volume frequency distributions to a changing community.

(7) The design and construction of a pedostat (a machine for carrying out continuous culture experiments on intact soil samples) is described.

(8) The oscillatory nature of microbial activity in a continuously stimulated model soil is described and discussed. Rate of CO_2 production was found to

oscillate between 700 and 57 nanomoles /g soil/hr with a decaying period. An unsuccessful attempt to achieve an oscillatory equilibrium in activity is described.

(9) Experimental verification of the applicability of a unitisation hypothesis to soil microbial communities is discussed. The unitisation hypothesis took the form of an empirical generalisation of the common features of all functional biological systems.

(10) Possible future extensions of the methods developed in the study are discussed.

(11) A conceptual model of the dynamics of a soil microbial community is presented. This encompasses the effects of physical, chemical and biological soil factors on the microflora and includes the effect of canopy activity and climatic variation. The model is based on an integration of the findings of this study and the literature discussed.

"Ecology has sometimes been defined as that branch of biology entirely abandoned to terminology. Ecology can also be defined as an art; specifically, as the art of talking about what everybody already knows about in a language that nobody understands. Microbial ecology has sometimes appeared to be the art of talking about what nobody really knows about in a language that everybody pretends to understand. The challenge in microbial ecology is to seek out the factors determining the growth of micro-organisms in their natural habitats and to talk about these factors in a language that everybody can understand."

(F. E. Clarke, 1967c)

1 SOIL AS AN ENVIRONMENT FOR MICRO-ORGANISMS

In order to appreciate the significance of the relationship between the properties of a soil and its microflora, it is necessary to understand the role of soil in the function of an ecosystem. An ecosystem can be considered as a self-perpetuating biological machine which converts radiant energy to chemical bond energy and subsequently releases it over a period of time as thermal energy. These two energy transformations are effected by autotrophs and heterotrophs respectively. In terrestrial ecosystems, the heterotrophic component ultimately responsible for thermogenesis is the soil microflora. This can be considered as the terminal component of the decomposer community (p 26) which itself

consists of a series of organisms comprising the complete spectrum of heterotrophic complexity from the simplest viroid to Homo sapiens.

Microbial thermogenesis is effected by degradation of substrates originally elaborated at the expense of solar energy by autotrophs. Complete mineralisation of substrates results in regeneration of inorganic nutrients from which autotrophs can elaborate further substrates. The processes of elaboration and mineralisation take place simultaneously and in an ecosystem in which the biotic and abiotic components are at equilibrium, a climax community exists in which there is no net organic matter accumulation. This means that inorganic nutrients are continously recycled and energy loss by thermogenesis is balanced by energy fixation in photosynthesis. Short-term fluctuations around this equilibrium are inevitable and analysis of equilibria can only be contemplated when an ecologically realistic time-scale is considered.

The function of the soil microflora is summarised in Figure 1 in which the progress of a substrate entering the soil can be traced. Where pathways fork, substrates (or their metabolites) are divided; the relative proportions which travel along each pathway depend on the dominant physico-chemical and biological conditions, which vary with time, and as a result of climatic factors. It can be seen that bond-energy in substrate molecules must travel around the main circular pathway a number of times in a variety of forms before it is ultimately released. Its progress is markedly affected by the abiotic components of the system.

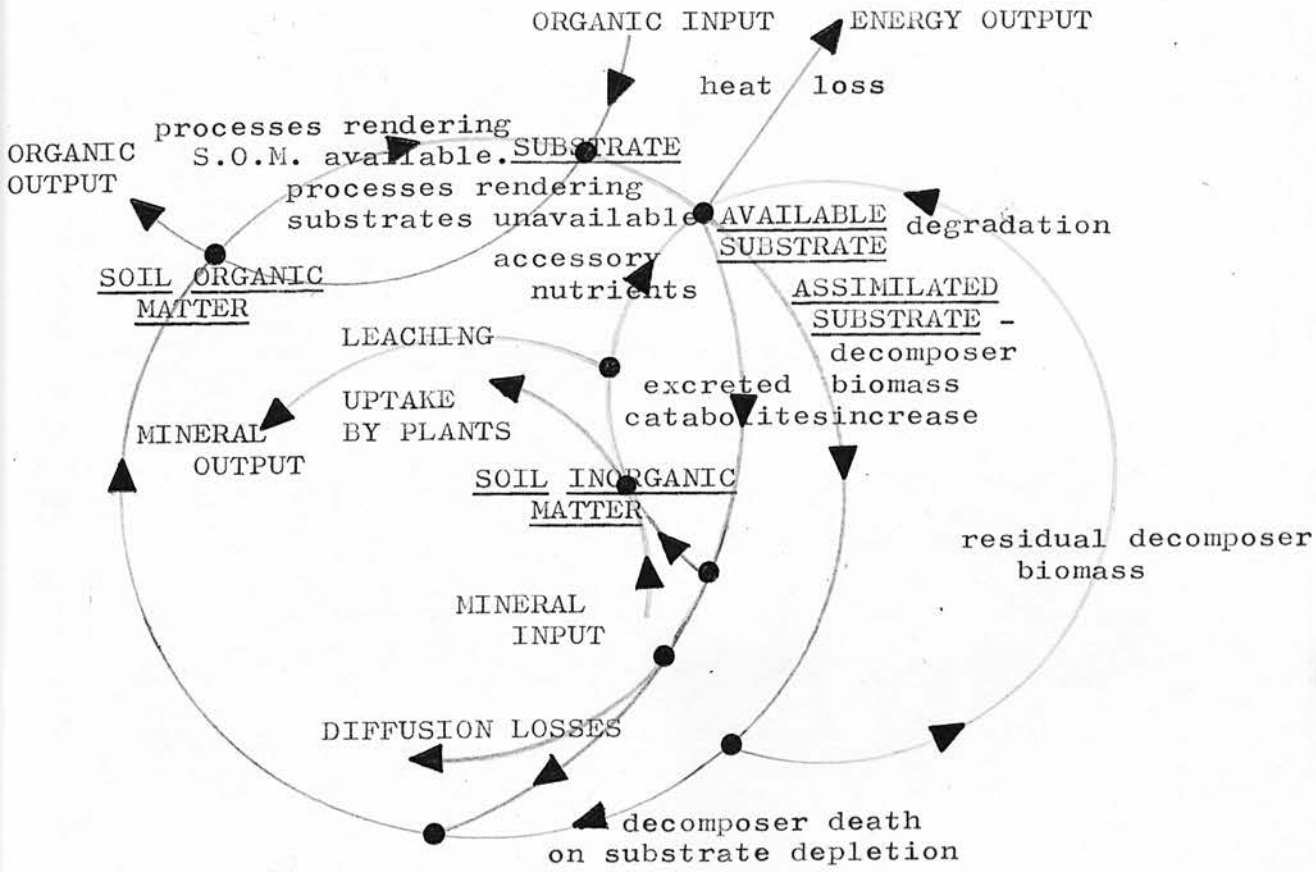


Figure 1. Function of the soil microflora in mineralisation of energy-yielding substrates.

(a) Effects of the physical nature of soil on micro-organisms

Soil is a three-phase system in which the solid phase is porous; the gas and liquid phases are distributed throughout the pores according to their relative concentrations. The physical attributes of soil which directly affect its microflora are three: structure, moisture and temperature. Aeration is sometimes included in this list (e.g. Raney, 1965.), but since its effects on micro-organisms are directly chemical, it will be used as a convenient link in the discussion of the effects of physical and chemical soil properties on micro-organisms.

(a) 1 Effects of soil structure on the microflora.

Micro-organisms in soil are probably predominantly in an adsorbed condition (Quastel and Scholefield, 1951; Marshall, 1971.). It has been demonstrated that the growth of bacteria in a solid/liquid system containing a low concentration of nutrients is enhanced by increasing solid surface areas. Zobell (1943) concluded that besides concentrating nutrients by adsorption and providing a resting place for sessile bacteria, solid surfaces retard the diffusion of exoenzymes and hydrolysates away from the cell, thereby promoting assimilation of nutrients which must be hydrolysed extracellularly prior to ingestion. Sorptive interactions between micro-organisms and solid-phase soil components have been reviewed by Marshall (1971). Although early studies of microbial adsorption on soil particles indicated that the phenomenon was disadvantageous to the organisms concerned (Khudyakov, 1926; Karpinskaya, 1926; Peele, 1936.), such conclusions were questioned by later workers Dikusar, 1940; Winogradsky, 1948; Zvyagintsev, 1959.). Recent evidence that cells are able to multiply quite readily in the

adsorbed condition and that growth is often stimulated by solid particles is well documented (Marshall, 1971).

It may be concluded that in gross terms, concentration of nutrients at particle surfaces favours readily adsorbed micro-organisms and while local adsorption of inhibitors such as antibiotics may provide protection for the producers, it will have a selectively detrimental effect on already adsorbed cells (Pinck et al, 1961; Soulides et al 1961, 1962). The significance of antibiotic production in soil has been reviewed by Brian (1957) and Park (1967).

The molecular level effects of microbial adsorption have been studied in terms of changes in enzyme activity with pH, primarily at cation-exchanging surfaces such as exist on clay particles. Elevated pH maxima for adsorbed enzymes such as chymotrypsin (McLaren and Esterman, 1957), phosphatase (McLaren and Skujins, 1967) and urease (Durand, 1964) have been demonstrated and interpreted in terms of the ionic double layer surrounding the clay particles. A similar explanation was offered for observations of elevated pH optima for Escherichia coli and Azotobacter agilis when adsorbed on anion exchange resins (Hattori and Furusaka, 1959a,b, 1960, 1961).

The variable effects of clays in enzyme inhibition have also been extensively studied. Enzyme adsorption to clays occurs by ionic bonding and by binding by non-coulombic forces (Aomine and Kobayashi, 1964). In the former case adsorption is related to the cation exchange capacity of the clay; in the latter case, adsorption depends on clay

surface area. The mechanism of inhibition has not been defined, although distortion of the enzyme molecule provides a possible explanation. Little information is available on local adsorption of enzyme inhibitors. Adsorption in most cases leads to irreversible reduction in activity.

Durand (1963, 1964a,b) noted partial inhibition of uricase after adsorption on bentonite; the activity of urease on adsorption was little affected while bacterial degradation of organic nitrogen was inhibited differentially in the presence of bentonite. Aomine and Kobayashi (1964, 1966) found that the three clay minerals allophane, montmorillonite and halloyisite differed in their inhibition of different enzymes, the order of inhibition of β-amylase being montmorillonite$>$allophane$>$halloyisite while that of a protease was allophane$>$montmorillonite$=$halloyisite. Ambroz (1966, 1968, 1970) has investigated the relationship between soil structure and the activity and distribution of proteases. While providing some degree of structural protection, adsorption often reduced activity, the extent of the reduction depending on the predominant clay mineral.

It has been proposed (McCalla, 1940a) that soil bacteria obtain their mineral requirements by contact exchange of adsorbed ions between the bacterial surface and clay particles. The cation-exchange capacity of the bacterial surface has been studied by McCalla (1940a,b). He showed that the order of the adsorption series was Na^{+}, NH_4^{+}, K^{+}, Mg^{2+} Ca^{2+} Ba^{2+} Mn^{2+} Fe^{3+} H^{+}. Adsorption and orientation of clay particles of sub-microbial size has been studied by Lahav (1962) and Marshall (1967, 1968, 1969). The latter author showed that differences in adsorption of Na^{+}-montmorillonite and Na^{+}-illite between various species of bacteria

could be interpreted in terms of the relative predominance of amino and carboxyl functions on the cell surface. Archibald, Armstrong, Baddiley and Hay (1961) suggested that teichoic acids in gram positive cell walls were involved in ion exchange. Recent work has substantiated this suggestion with particular reference to divalent cations such as Mg^{2+}. (Heptinstall, Archibald and Baddiley, 1970; Hughes, Hancock and Baddiley, 1973). The amount of Mg^{2+} bound is related to the alanine ester (Archibald, Baddiley and Heptinstall, 1973) and phosphate content (Ou, Chatterjee, Young and Marquis, 1973) of the teichoic acid.

Adsorptive surface in different soils varies quantitatively with particle size and compaction and qualitatively with the nature of the soil colloids. The internal area of soils is prodigious: 1 ft^3 of heavy clay loam has an internal area of 22.7 acres (Chapman, 1965).

(a) 2 Interactions between soil moisture and the microflora.

Water is a major constituent of protoplasm and a solvent for all nutrients. Regardless of habitat, micro-organisms are primarily aquatic (Stotzky, 1973). Those organisms which can traverse air-filled pore space in soil e.g. actinomycetes and fungi have been regarded as being adapted to terrestrial conditions (Bisset, 1963). However, their cell surfaces are surrounded by a water film (Stotzky, loc cit) continuous with adjacent soil aggregates. Hence this situation is an extension of the aquatic environment. In the case of micro-organisms possessing airborne stages in the life-cycle, some degree of independence is gained from the aqueous environment. In these cases it is the dispersal organs which are dessication-resistant.

Perhaps the extreme case of cell division at low water activity (defined below) is that reported for Pleurococcus vulgaris (Zeuch, 1934) at a_w 0.48 at 20°C. The extremity of this situation is emphasised by McLaren and Skujins (1967) : "at this relative humidity a typical protein contains about 8% water and enzyme reactions must take place in the absence of a liquid water phase at very low velocities." Availability of water to micro-organisms is usually expressed as the limits of water activity (a_w) between which a specific function takes place (Stotzky, 1973).

$$a_w = Ps/Pw = RH/100 \qquad (1)$$

where Ps is the vapour pressure of the solution, Pw is the vapour pressure of pure water at the same temperature and RH is relative humidity. Much data has been accumulated from in vitro studies of a_w requirements of micro-organisms and microbial functions (Griffin, 1963; Rose, 1965). Minimal a_w values of 0.85 - 0.95 for fungi and ca. 0.93 for bacteria are general. Most microbial functions take place maximally at higher a_w e.g. for most fungi, growth decreases by half at a_w 0.94 - 0.97 compared with that at a_w 1. Survival and growth of micro-organisms at different a_w is correlated with taxonomic status and possession of spores of various kinds. The application of such studies to micro-organisms in soil is difficult because of the nature of soil water. Most field or laboratory data refer to growth at a specific percentage of the soil's water holding capacity (WHC).

Early work in this field indicated that microbial growth and activity was maximal between 60 and 80% MHC (Chase and Gray, 1957; Kononova, 1961; Russell, 1961). Greaves and Carter (1920) studied 22 soils

which differed structurally and chemically. They found that nitrification and ammonification were maximal at ca. 60% MHC while nitrogen fixation was maximal between 70 and 80%. The elevated moisture content in the case of nitrogen fixation would be associated with an increased number of anaerobic or partially anaerobic microhabitats (Greenwood, 1967) which agrees with the established oxygen sensitivity of nitrogenase. Kostychev (1886) showed that maximum organic matter decomposition occurred between 60 and 80% MHC (Kononova 1961). Swietochowski and Miklaszevsky (1962) have largely confirmed the results of these early investigators.

The survival of micro-organisms in dry soils is well known. Anthrax was reproduced in guinea pigs inoculated with cultures from soil collected at the Lacrosse anthrax outbreak of 1902. The soil had been air-dried and stored for 60 years at room temperature (Wilson and Russel, 1964). Survival is not restricted to endospores. Nitrosomonas can be recovered from air-dried soils after several years (McLaren and Skujins 1967). The survival of micro-organisms is predictably varied: Rhizopus nigricans showed high survival capacity while low numbers of Fusarium oxysporum were recovered from 5-year old air-dried soil cultures. (Atkinson, 1954). Soil algae were also recoverable from soil stored air-dry for 26 - 73 years in a hermetically sealed condition (Bristol, 1919). Such survival phenomena are probably related to the great tenacity with which soil retains the last traces of its moisture complement. For example, the relative humidity of desert soils has been found to be rarely less than 85% at the surface where the RH of the atmosphere was less than 15% (Cameron, 1966).

Aknowledge of soil moisture dynamics is essential if the complexities

of microbe/soil/water interactions are to be appreciated. A good introductory treatment is that of Kohnke (1968).

Because of the aggregated structure of soil, liquid soil water takes two main forms; that contained in macropores between aggregates (gravitational water) and that in intra-aggregate micropores (capillary water). The greatest amount of water which a soil can retain against the force of gravity is controlled by porosity and is known as the moisture holding capacity. Thus large aggregate soils with high porosities are freely permeable to water whereas silts and clay soils are not. Capillary water in soil obeys the laws of surface tension, capillary rise, capillary retention etc. familiar from classical physics because the soil solids are hydrophilic. Soil solids are normally largely inorganic and dominated by silicon, aluminium and oxygen. Their external atoms are frequently oxygen which have unshared electrons free for hydrogen bonding to water dipoles and electrostatic adsorption of charged organic materials. Soil particles tend to have a superficial negative charge and behave as ions towards water dipoles, attracting them with a force 10,000 times as great as between-dipole hydrogen bonds. The forces between soil colloids and water are governed by an inverse square law; though it is possible to remove some of the capillary water by the application of suction, the last traces are so firmly bound that extremely strong forces must be applied to remove them.

In physical terms, after the removal of most of the capillary water, some remains on surfaces because of adsorption and solution in humus (hygroscopicity). Osmotic reduction in vapour pressure also plays a

role in minimising moisture loss. Moisture exists in the vapour phase in those pores which are not water-filled.

Because of the complexity of soil moisture relationships, the choice of a moisture parameter in studies of soil microbial acticity is difficult. To utilise water, an organism must exert the energy necessary to remove it from the soil. This energy has been expressed in many ways (moisture tension in atmospheres or millibars, equivalent water column height in cm etc.) and a correlation of these parameters is presented in Table 5 (p.132). The amount of energy may be expressed logarithmically by the pF scale (Schofield, 1935):

$$pF = \log (RT/Mg \times \ln P/P_o) \qquad (2)$$

R = gas constant; T = absolute temperature; M = molecular weight of the liquid; g = gravitational constant; P/P_o = relative vapour pressure.

To remove water from a 1.5 µm diameter pore, a suction of one atmosphere (1,000 cm of water, pF 3) is required. Under these conditions, micro-organisms can easily compete with plant roots (McLaren and Skujins 1967).

(a) 3 Soil/microflora/temperature interactions

Microbial metabolism is governed by the laws of thermodynamics (Farrel and Rose, 1967; Stotzky, 1973); consequently physiological reaction rates are a function of temperature. In addition to controlling the activity of the soil microflora, the temperature regime of the environment can affect community structure. Such effects are noticeable only

in extreme situations such as hot springs (Brock and Brock, 1968), compost heaps, arctic soils etc. The optimal growth temperature for soil micro-organisms varies between 20 and 35°C (Brock, 1966; Clark, 1970). Most organisms grow well at 10°C and more slowly at lower temperatures (Clark, 1967a). Distictly tropical and temperate soil microfloras are not recognised. The microflora of an antarctic soil differs from that of a comparable temperate moorland in terms of the cold-tolerance of only a proportion of the micro-organisms present (Heal, Bailey and Latter, 1967) i.e. the majority of microbial species are relatively ubiquitous.

The two sources of temperature fluctuation (diurnal and seasonal radiation cycles) may have quite different effects on the microflora. There appears to be no readily demonstrable effect of diurnal temperature fluctuations on soil micro-organisms. This may be attributed to the buffering effect of the soil (Kniebe and Kopf, 1970) and the long generation times observed in soil. The average thermal capacity of soil forming minerals is ca. 0.2 cal/g/°C while the thermal capacity of water is defined as unity; i.e. the rapidity of temperature change in soil is controlled by the moisture content. Daily soil temperature fluctuations are great only at the surface; at 10cm depth, they rarely exceed 4-5°C and at 30 cm 3°C at temperate latitudes (Kohnke, 1968). Fluctuations of lower amplitude have been reported by Kniebe and Koepf (1970) in a theoretical study of the interplay of soil thermal conductivity and amplitude of surface temperature fluctuations.

The effect of seasonal changes in temperature is likely to be indirect.

Where soil microbial numbers have been measured over extended periods, little correlation with temperature has been noted (e.g. Cutler, Crump and Sandon, 1922). Pronounced seasonal flushes of microbial activity are however well known, maximal activity occuring in spring and autumn. (e.g. Cutler *et al*, *loc cit*). A plausible explanation for this is that temperature and other seasonal climatic factors, by stimulating plant growth and senescence, indirectly affect the soil microflora by the provision of fresh nutrient materials. In the spring such materials may consist of sloughed root cells, mucigel, plant metabolites etc. In the autumn, dead leaves and roots provide nutrients. Although the role of the microflora in thermogenesis has been stressed (p.1), the rate of heat output is low and transitory local temperature differences of *ca*. $1^{o}C$ from this source are probably maximal (Clark *at al*, 1962). Temperature differences of this order have only been noted between islands of organic material and an otherwise mineral soil.

(b) Effects of the chemical nature of soil on micro-organisms

(b) 1 Aeration

The effects of the aeration status of soil upon the microflora are ultimately controlled by gas solubility and solution diffusion rates. Diffusion rates of gases in solution are commonly assumed to be of the order of 10^{-4} of those measureable in the atmosphere (Raney, 1965; Clark 1967). The most important dissolved gases for micro-organisms are oxygen and carbon dioxide. (Availability of N_2 for soil micro-organisms has been little studied (Shields and Durrell, 1964)). An unusual mechanism of oxygen supply to micro-organisms occurs in waterlogged soils (Bartlett, 1961; Armstrong, 1964) where certain plants by means of root aeration canals continuous with the atmosphere, can maintain an oxidising rhizosphere.

The nature of energy-yielding pathways in micro-organisms is governed by the availability of oxygen. Oxygen can have a direct effect on the efficiency of microbial thermogenesis: respired glucose yields 690 kcal/mole, 55% of this being released as heat, while glucose fermentation yields 58 kcal/mole of which 75% is lost as heat. Incomplete substrate oxidation resulting from fermentation leaves much bond energy locked in metabolites. The diversity of metabolites produced in fermentation has been postulated as a selection mechanism governing microbial community structure i.e. species diversity (Stotzky, 1973).

In many soils, the changeover from aerobic to anaerobic metabolism occurs at oxygen concentrations of ca. 3×10^{-6}M (Greenwood, 1961; Greenwood and Berry, 1962). Because of the biochemical oxygen demand

of colonised aggregates, moisture saturated soil crumbs of radii greater than 3mm have completely anaerobic centres i.e. at this limiting size micro-organisms can remove oxygen more quickly than diffusion can replace it. This can provide one explanation for the universality of strict anaerobes in apparently aerobic soils.

The inverse dependence of air-filled pore space on moisture content and the localised effects of diffusion emphasise the importance of soil moisture on microflora/soil atmosphere interactions. Despite Stotzky's (1973) opinion that "although diffusion occurs, it is too slow a process to account for much of the gas exchange", it is an accepted fact of soil physics that diffusion is the most important means of renewal of soil air (Kohnke, 1968). Mass flow of the soil air (the only alternative means of renewal) is convincingly dismissed by Kohnke (loc cit).
In soils with porosity greater than 10% by volume, the rate of exchange of oxygen and carbon dioxide with the surface atmosphere comes to equilibrium to a depth of 30cm in ca. 1 hour (McLaren and Skujins, 1967).

In the surface layers of a well aerated soil, oxygen concentration in the gas phase is 18 - 21% v/v; carbon dioxide at 0.1 - 5% (maximally 20%) is always more concentrated than in the external atmosphere.
When soil moisture tension is low and localised reducing conditions occur soil air is enriched with metabolic products such as CH_4, H_2S, NH_3, N_2O and C_2H_4. Volatile metabolites can provide chemotactic stimuli for soil micro-organisms (Stotzky, 1973) or act as inhibitors (Parr and Reuszer, 1962).

The effects of high CO_2 concentrations in the soil air are diverse. Nitrification is directly stimulated in the presence of adequate

oxygen.(Clark, 1967b; Harmsen and van Schreven, 1955; Beard and Clark 1962). Inhibition of portions of the microflora by excess CO_2 has also been indicated (Stotzky and Goos, 1966). Because of the greater concentration of micro-organisms and their metabolites in soil, CO_2/ HCO_3^- pH buffering systems are of less consequence than in aquatic systems.

The interpretation of redox potential as an environmental parameter is surrounded by considerable uncertainty. Apart from assuming a direct effect on metabolic redox couples, it is best to take the view of McLaren and Skujins (1967) that "... it is not yet clear whether the redox potential per se is important for microbial activity or if it is only a reflection of oxygen availability and the influence of oxidising or reducing compounds on micro-organisms." A classical treatment of the relevance of E_h to micro-organisms is that of Hewitt (1950); a more recent study is reported by Wimpenny and Necklen (1971).

Until more is known of the effects of soil aeration status at the micro-environment level, the optimal soil moisture level for microbial activity must be considered to be that compatible with optimal aeration for the bulk of the microflora (Clark, 1967c).

(b) 2 Inorganic nutrition of soil micro-organisms.

The role of ion-exchange in cation uptake by micro-organisms has already been mentioned (p. 5,6). The concentrations of cations necessary for microbial growth are regulated in soil by equilibria depending on the bulk (liquid phase) pH and the dissociation constants of acidic groups in the soil colloids.

In continuous culture work, the importance of the limiting nutrient in controlling microbial growth is common knowledge; in soil studies the same concept is involved in the use of substrate carbon:mineral ratios. The emphasis is normally on organic matter degradation; this takes place increasingly rapidly as relative mineral concentrations are increased. Wide variations in carbon:mineral ratios have been noted (Bremner, 1967); such ratios are probably the most useful indicators of the gross microbiological status of a soil. In agriculturally well balanced soils there is a tendency towards uniformity in carbon:mineral ratios. The average carbon: nitrogen: sulphur :organic phosphorus ratio from 20 New Zealand soils was 120:10:1.3:2.7 (Walker and Adams, 1958); a similar average of 140:10:1.4:2.4 has been obtained for 50 Scottish soils (Williams, Williams and Scott, 1960). The similarity of such ratios to the elemental composition of bacteria has been noted (Alexander, 1961). Immobilisation of various minerals in microbial tissue is likely to take place in a linked manner; i.e. different minerals will disappear from the soil at rates which are approximately constant relative to one another.

The nature of biogeochemical cycles in natural systems is so well known that it requires no emphasis (see for example, Alexander, 1961; Odum, 1969). At the organismic level in soil, the factors of importance in determining the availability of the mineral ions required by microorganisms have already been discussed. The extent of these factors is determined by the chemical nature of minerals in soil. After carbon, nitrogen is the most important element in microbial nutrition. It is surprising that until recently, it was assumed that most soil nitrogen was organic, mineral nitrogen rarely exceeding 2% (Bremner,1967).

Recent studies have indicated that 5 - 50% of soil nitrogen can be in the form of clay-fixed ammonium. The ammonium is non-exchangeable and its availability to micro-organisms is unknown. Nitrate is generally considered to be an ephemeral soil component; its production depends on the activity of specific components of the microflora and as a nitrogen source for higher plants, it has a short residence time in soil. Low detectable nitrate concentrations do not preclude high nitrate turnover between nitrifiers and higher plants. Nitrite and esoteric nitrogen compounds (hydroxylamine, hyponotrous acid, imidonitric acid and various nitrogen oxides) implicated in microbial nitrogen transformations are largely undetectable in soils.

Phosphorus and sulphur follow nitrogen in the list of mineral nutrients important to micro-organisms. It has been reported that 1/2 - 2/3 of the total phosphorus (Cosgrove, 1967) and almost all the sulphur in soil is organic (Freney, 1967). The sulphur data is of restricted value since knowledge of soil sulphur is limited. The organic nature of soil phosphorus and sulphur may be correlated with the low level of anion exchange activity found in soils; i.e. anions are fixed much less readily than cations and dissolved concentrations are dependent on equilibria between microbial degradation of organic material, plant uptake and leaching.

(b) 3 Supply of energy- and carbon-yielding substrates.

50 - 95% of soil nitrogen is normally organic; total soil nitrogen ranges from less than 0.02% in subsoils to greater than 2.5% in peats (Bremner, 1967). The concentration in soil is determined by the following factors (in order of importance): climate, vegetation, topography, parent material, age (Jenny, 1930). 20 - 50% of the total nitro-

gen in most surface soils is in the form of bound amino-acids and 5 - 10% is in the form of bound hexosamine. Purine and pyrimidine bases make up less than 1% of the nitrogen in most soils (Bremner, 1967). Although the amino-acids and amino-sugars extracted from soil can be identified chromatographically, the nature of materials from which they were hydrolysed and the non-hydrolysable nitrogen residue is unknown.

5 - 16% of soil organic matter is carbohydrate (Gupta, 1967). The function of carbohydrate gums, mucilage and microbial slime in stabilisation of soil aggregates is well established. As substrates for the microflora, their availability is governed by their reactions with clay and humic components; physical non-availability (i.e. adsorption in pores of submicrobial size) may also be important (Allison, 1947; Russell 1961). Hydrogen bonding can take place between sugars, polysaccharides and clays, adsorption possibly taking place on the interlamellar surfaces of the clay crystals (Greenland, 1956). The carbohydrates contained in soil are mainly polymers of hexoses, pentoses, methylated sugars, uronic acids and some unidentified sugars (Gupta, loc cit). Glucose constitutes a major proportion of the total carbohydrates, remaining so in all horizons while total carbohydrate concentration tends to decrease with depth in most soils. Cellulose accounts for 8-14% of soil carbohydrate; free monosaccharides account for less than 1% of the total but this low level may represent an equilibrium between dissolution and utilisation.

Although most of their monomer components have been identified, the exact source and structure of ca.70% of soil carbohydrates is still obscure. The role of micro-organisms in the production of carbohydrates has already been indicated. The nitrogenous polysaccharide chitin is

of exclusively fungal and faunal origin while much of the cellulose, hemicellulose and polyuronic acid is probably plant-derived. An introduction to soil carbohydrates is given in the review of Gupta (loc cit); a more extensive treatise on the chemistry and biochemistry of soil polysaccharides is provided by Finch, Hayes and Stacey (1971).

In addition to carbohydrate materials, the major plant-derived substrate for microbial growth is lignin. Because of certain qualitative similarities and a probable genetic relationship, it is logical to discuss lignin and humic materials together (Hurst and Burges, 1967). Neither of these two substances can be considered as a distinct molecular entity even though the composition of lignins is much less variable than that of humus. The basic structure in lignin complexes is the phenyl propane skeleton (Schubert, 1965); lignin is a polymer of such subunits bonded largely by aryl ether cross-linkages.

The nature of the cyclic part of the phenyl propane unit is indicative of the type of plant from which the lignin is derived. Vanillin and some p-hydroxybenzaldehyde are obtained from coniferous lignin; vanillin and syringaldehyde come from woody dicotyledons; all three of these phenols are obtained from monocotyledons. (Hurst and Burges, 1967). Thus it is possible that the chemical nature of the canopy may select for specific micro-organisms in the soil on the basis of their ability to degrade specific phenols.

In soil, most lignin is in a finely divided form. Since the bulk of the work on lignin degradation has been done on white-rot fungi in commercial timber, (Schubert, 1965), little is known of the degradation and availability of natural lignin in soil. Some of the material is certainly degraded, since lignin per se is

not considered to be a specific component of soil organic matter. The more or less intractible remains are thought to undergo polymerisation with polyphenols, proteins, carbohydrates and monophenols at least some of which are microbial metabolites (Hurst and Burges, 1967; Oglesby et al, 1967). The resulting complex is known as humus. Within this context, the isolation from pure culture filtrates of humus-like polymers is currently popular (Robert-Gero et al, 1967; Haider and Martin 1970; Matschke and Igel, 1971). The highly variable origin and constituents of the polycondensate known as humus naturally preclude rapid enzymic degradation. Structures which once served precise functions have lost this reason for existence and it has been suggested by Bacon (1967) that little of biochemical significance can be expected to remain. None the less, humus is degraded in all mineral soils. Conversely in peat soils, organic matter accumulation takes place, gradually leading to a more and more oligotrophic soil as minerals are progressively immobilised in undecomposed plant material. Humic materials also serve a potentially important role in cation exchange and immobilisation in mineral soils (Broadbent and Bradford, 1951) having an exchange capacity about twice that of clay minerals (Chapman, 1965).

Humus occurs to different extents depending on soil type: ca 1.5% in a sandy loam to ca. 9.2% in a heavy clay loam (Robinson, 1932). As well as those organic materials already considered, there exists (at least temporarily) in soil the complete range of biochemicals elaborated by higher plants and members of the decomposer community. Although some of these such as plant hormones, vitamins and antibiotics are so dilute that they are difficult to detect, they are

biologically effective at very low concentrations and may therefore be of importance to soil micro-organisms at the micro-environment level. In this context it is of interest to note that rainwater has been cited as a significant source of vitamin B12 (Parker, 1968). In oligotrophic soils, rainwater is also a significant source of minerals (Leyton et al, 1967; Gore, 1968; Allen et al, 1968). In certain habitats it is the sole source of mineral input, e.g. blanket peat habitats in the Northern Pennines (Crisp, 1966). Perhaps the ultimate source of energy- and carbon-yielding substrates is provided by the soil microflora itself (Tribe, 1963). Since utilisation of other substrates eventually necessitates the reproduction of microbial biomass, some mechanism for the degradation of microbial residues exists in soil since cells do not accumulate indefinitely. This subject has been adequately reviewed by Webley and Jones (1971).

(b) 4. Consideration of the nature of the micro-environment of soil micro-organisms.

Theoretical studies on the effects of soil structure at the organismic and micro-colony level are few. The work which has been done can be understood on the basis of material considered in the preceding discussion. On the basis of physical measurements (largely on the stability of soil crumbs under various conditions of dispersion) Emerson (1959) elaborated a general model of soil crumb structure. The model appears to be applicable to a wide range of soils and does not contradict experimental data on soil behaviour related to aggregate stability. The two main constituents of soil are quartz and clay; these components bonded together

represent the elementary unit of soil structure - the crumb, or aggregate. Emerson's experiments and theories provide valuable information on the way the soil components are bonded together and on the possible effect of this on micro-organisms. The hypothetical soil crumb is shown in Figure 2 (overleaf). The flat clay crystals are considered to be oriented into domains which behave in water as a single unit. Domain synthesis is easily explained in terms of normal cation saturation and drying regimes experienced in agricultural soils. The domains are bonded to the quartz particles and one-another by films of organic material. Domains may also be electrostatically bonded as a result of crystal edge/face charge opposition as indicated in Figure 2. The possibility was noted by Emerson (loc.cit.) that in many soils the clay crystals may be segregated into domains pure with respect to specific clay minerals. The nature of the quartz/clay bond would depend to some extent on the nature of the organic material. The ease with which bonds are broken, and the tetrahedrally linked silicon surface of the quartz grains indicates that they are primarily hydrogen bonds. In some soils however, sesquioxide coatings on the quartz may strengthen the quartz/clay domain bonds.

The parts of this model which are of greatest importance to micro-organisms are the size of the aggregate, the size of the pores it contains, whether the pores are air or water filled, the nature of the clay and organic matter, the ease and regularity of aggregate disruption. Rather than the absolute size of aggregates which are almost certainly made up of a large number of the elementary units illustrated in Figure 2, the size of the

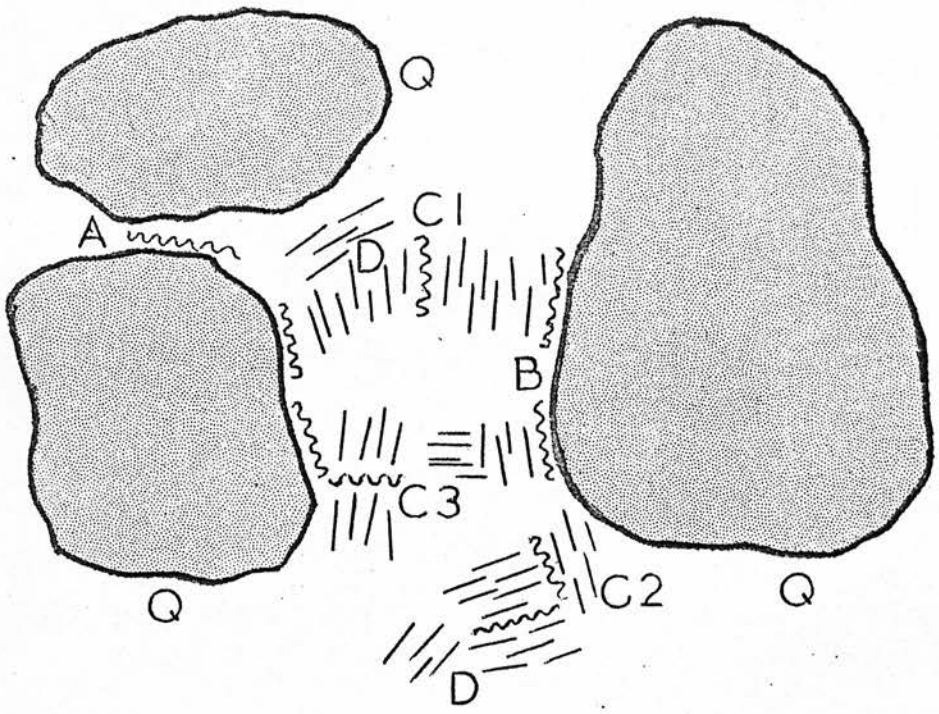


Figure 2. Possible arrangements of domains, organic matter and quartz in a soil crumb. (Modified after Emerson, 1959).

Types of bond:
- A. Quartz-organic matter-quartz.
- B. Quartz-organic matter-domain
- C. Domains-organic matter-domain
  - (1, face - face
  - 2, edge - face
  - 3, edge - edge.)
- D. Domain-domain, edge-face.

Q = quartz grains.

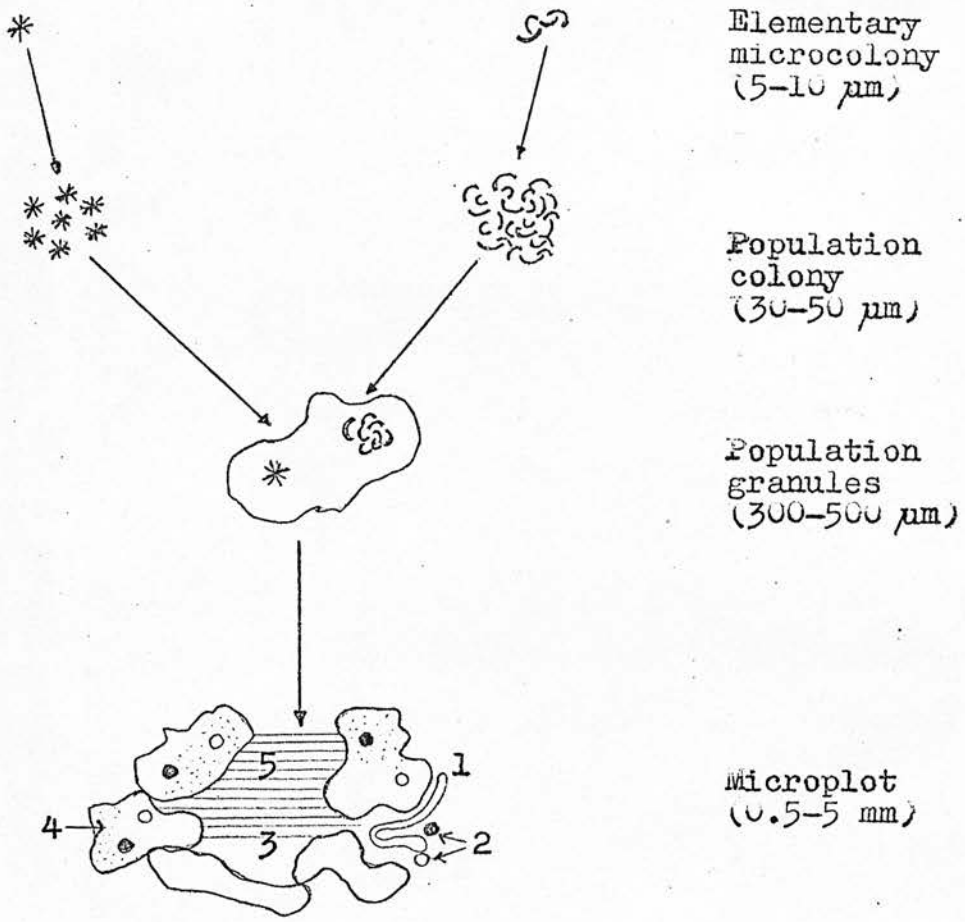
pores they contain is the parameter which will limit microbial colonisation. Since quartz particles range from coarse sand to grains of less than 2 μm diameter, the pore size distribution is one of the bases of soil texture and will vary with this parameter. The pore size distribution of a soil can be estimated experimentally on the basis of pF characteristics (p. 10, 132). Salter and Williams (1965) made such measurements on 18 soils of varying textural types and found that in all except one case, greater than 50% of the water was retained at a tension of 0.3 atmospheres. This indicated that most of the pores had a diameter of less than 5 μm. Since this is of the order of magnitude of single bacterial cells, decomposition of the aggregate-cementing organic material must be severely retarded by physical shielding. Enzyme adsorption by clays will also tend to preserve aggregate stability. The relationship of moisture and aeration status to aggregate size in terms of microbially induced anaerobiosis has already been discussed (p. 14).

From such considerations it can be seen that major sources of organic materials for microbial growth will be:

(1) islands of plant or animal derived material which have preserved some degree of structural integrity, and

(2) those forces which tend to cause aggregate disruption and the exposure of organic materials either to direct microbial attack or to unimpeded diffusion of extracellular enzymes. The stimulative effect of disruption of soil microstructure by physical means such as freezing and drought are well known (Nowak, 1965; Jager, 1967; see also p. 187). In such surroundings, the attraction of the rhizosphere as a

microbial habitat must assume large proportions. On the basis of microscopic examination Nikitin (1973) has tried to establish the existence of an elementary microbiological structural unit in soil. Although he uses the term 'elementary microbial ecosystem' to describe such a unit it should be recognised that as an essentially completely heterotrophic system, no part of the decomposer community can be considered an ecosystem. It is undesirable however to coin new terms and definitions at the drop of a hat and with this reservation noted, Nikitin's definition can be used unambiguously. According to this author, the primary structure of populations on natural substrates appears to be a 'micro-aggregate' made up of some dozens of cells. (All terms in inverted commas are Nikitin's). These 'micro-aggregates' or 'elementary microcolonies' are considered to be physically bound together by slime, fimbriae, stalks etc. and to reproduce to give multiple units ('population colonies') consisting of hundreds of cells. Nikitin regards such colonies as the terminal population unit in which cycles of growth, senescence and lysis take place. Combinations of such 'population colonies' and abiotic soil components give rise to 'population granules' which in turn combine to produce a soil crumb, or in Nikitin's terminology, 'microplot'. This scheme is illustrated in Figure 3. It is unfortunate that Nikitin does not appear to have considered the Emerson soil aggregate model; Nikitin's population sub-units would appear to be those confined to the external surfaces of comparatively large soil aggregates. In terms of surface area, this kind of habitat is probably a small proportion of most soils (p. 6). However, with the advent of a technique for floating off surface films of micro-organisms and clay particles



igure 3. The formation and structure of the elementary microbial 'ecosystem' according to Nikitin (1973).

1.Plant residue
2.Microbial microcolonies
3.Capillary
4.Pore
5.Native organic matter (humus)

from soil aggregates (Grossman and Lynn, 1967; Harris, 1972; Waid, 1973) studies of this nature should make rapid progress.

(c) Effects of the biotic environment on the activity of soil micro-organisms

The position of the soil microflora in the decomposer community can be assessed from the scheme outlined below. This has been compiled from various sources: Burges, 1956; 1965; Garrett, 1963; Frankland, 1966; Latter and Cragg, 1967 etc.

Condition of organic material		Associated decomposers
(1) living leaves, shoots and roots		parasites, endophytes, phyllosphere and rhizosphere microflora - fungi, yeasts, bacteria, protozoa
(2) moribund or senescent leaves and roots	CHRONOSEQUENCE	weak parasitic and early saprophytic fungi
(3) standing dead (i.e. material still attached to plants		early saprophytic fungi
(4) litter (L layer - recognisable plant detritus)		fungi (often dominant pH < 5.5), bacteria (often dominant pH> 5.5)
(5) litter (F layer - largely degraded)		mites, collembola, earthworms, enchytraeids (dominance habitat dependent), fungi, bacteria
(6) litter (H layer - microscopic fragments and faunal faeces)		fungi, meiofauna, bacteria
(7) humic materials dispersed in mineral soil		bacteria, fungi, protozoa.

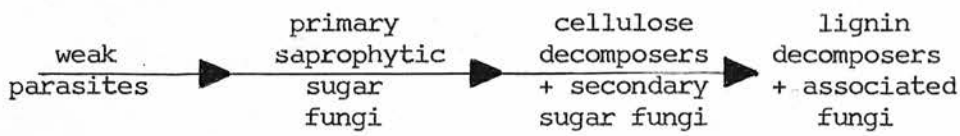
As in all ecological analyses, compartmentalisation gives a false impression of simplicity but can effectively freeze a dynamic system, allowing discussion of its components part by part. All the processes outlined above take place simultaneously and in many

terrestrial ecosystems are not spatially separated from one another. Because of their essentially opportunist natures, specific decomposer organisms can be expected to flourish wherever suitable nutrients and environments coincide. The fact that a recognisable sequence of colonisation and decomposition can be found indicates strong interdependence between these organisms, initial colonisers necessarily preparing the habitat for those which follow. This habitat preparation is closely linked with the ease or difficulty with which substrates can be degraded.

A substantial proportion of the more readily degradable tissue components is often lost before the material reaches the litter layers. Heavy colonisation of senescent Pinus sylvestris needles, Urtica leaves and stems (Burges, 1965) and Pteridium aquilinum petioles (Frankland, 1966) have been noted (stage 2, p. 26). Similar colonisation of grasses (Webster, 1956, 1957), a sedge (Pugh, 1958) and the leaves of trees are also well known. Experiments have been carried out on the change in chemical composition of various litters and on the accompanying microbiological changes. The precise course of decomposition appears to be governed by climate and the physical and chemical nature of the plant material.

In pine litter (Kendrick and Burges, 1962) much of the chrono-sequence is preserved in a spatially intact form since larger members of the soil fauna do not appear to be active in litter comminution. The primary fungal invaders are associated with a needle dry weight loss of ca. 20%. After this stage, the degradation rate drops and the grazing action of mites (and to a lesser extent springtails) on the hyphae and spores assumes importance. Endophagous mites then invade the interior of the

needle, eventually reducing most of it to a loose mass of faecal pellets derived from plant and hyphal material. This animal phase in organic matter degradation is judged to be essential (Burges, 1965) and has also been noted in dung and grass decomposition. During the primary fungal invasion, readily fermentable materials such as starch, hemicellulose and pectins disappear and protein is partly removed. After the animal stage, an intense wave of basidiomycete development takes place; this is correlated with the disappearance of most of the cellulose and lignin. As the basidiomycete biomass increases, so again does that of small soil animals, their combined activity being such that the H layer (stage 6, p. 26) becomes enriched in fungal and micro-arthropod skeletal material and faeces. It would appear that at this stage there has been very little nitrogen loss, the plant derived nitrogen being immobilised in microbial tissue and debris. Loss of other mineral ions by leaching in a similar system appears to take place much more rapidly (Burges, 1956). The decomposition series described above fits into the classical fungal sequence described by Garrett (1963) for the decomposition of plant material in or on the soil:



This pattern is not however universal, and in cases where the composition of the plant material is greatly different from that described above, the succession can be abbreviated or reversed. A reversed succession was found by Frankland (1966) on decaying bracken petioles. This highly lignified substrate was initially colonised before frond-fall and readily soluble material was removed.

The process of humification goes on continuously in all the stages of decomposition discussed; however at the end of this succession the most assimilable substrates are in the form of micro-arthropod faeces and corpses and fungal debris. It has been calculated by Dudich, Balogh and Loksa (1952) and Nef (1957), that all the annual litter fall in woodlands is eaten eventually by the soil fauna. Litter fall can vary from 1.5 tons/acre in temperate hardwoods to greater than 20 tons/acre in tropical rain forest (Burges, 1965). Since greater than 60% and usually 90% of the food litter is returned to the soil as faeces (van der Drift, 1951; Gere, 1956, 1962; Bocock, 1963) substrates in this form must be of tremendous importance to micro-organisms in such environments. Very little information is available about faecal pellet decomposition (Parle, 1963; Nicholson, Bocock and Heal, 1966). The studies of Nicholson et al (loc. cit.) on the faeces of the millipede Glomeris marginata (Villers) showed that a succession of micro-organisms quite different from that decomposing the parent plant litter (hazel leaves) took place. Early loss of carbohydrates was correlated with high respiratory quotient and maximal bacterial counts. Fungal counts increased as the pellets aged; Phycomycetes grew strongly as initial colonisers and were rapidly replaced by Fungi Imperfecti and Ascomycetes. The decomposition process and others described earlier lead to significant chitin accumulation from mycelial walls and micro-arthropod exoskeletons. The breakdown of this substrate has been studied principally by Okafor (1964, 1965, 1966, 1967). Chitin appears from these studies to support a highly varied microflora consisting of a number of genera of fungi, eubacteria, actinomycetes, protozoa and nematodes.

Throughout this discussion of decomposition, examples have recurred in which complex mixtures of substrates were rendered progressively more difficult to decompose as a result of the early removal of the more assimilable components. Thus from a variety of sources, a list of litter and soil components in order of degradability (or preferability) can be constructed:

1 sugars, starches, amino-acids, soluble proteins, short-chain fatty acids;

2 some high molecular weight proteins;

3 hemi-celluloses, pectins;

4 cellulose;

5 lignin, lignin-protein complexes, oils, fats, waxes;

6 humic complexes.

An attempt has been made to assess the 'assailability' of litter components by chemical means (Minderman, 1967). The analyses cannot however be expected to use the same discriminatory criteria as the micro-organisms. The property of ease or difficulty with which a substrate may be degraded has been christened 'molecular recalcitrance' (Alexander, 1965). This author has discussed the subject at length and has provided a list of reasons to explain the failure of micro-organisms with respect to the degradation of recalcitrant substrates. Most of these mechanisms which are of relevance to normal (i.e. non-polluted) soil microbial activity have already been considered. In the context of litter decomposition, tannin inactivation of enzymes should be mentioned (Basaraba and Starkey, 1966; Benoit and Starkey, 1968a, b; Benoit et al, 1968); this can be a significant factor in cellulose breakdown. Inhibition of microflora activity by other water soluble materials derived from

litter or foliar autolysates has also been noted (Beck et al, 1969; Boquel et al, 1970; Andreux et al, 1970). In soil where inhibitors may well become part of the humic complex, a reversible competitive inhibition of added pronase by humic acid has been noted (Ladd, Brisbane and Butler, 1968).

In the face of the known recalcitrance of humus and the many mechanisms which may physically or chemically prevent its breakdown, the versatility and persistence of the sub-terranean microflora is surprising. It is generally assumed that the soil microflora (given time) is capable of degrading any organic material of biological origin (Alexander, 1965). In experimental systems, the addition of a readily assimilable substrate gives rise to an immediate flush of activity - the familiar zymogenous response. If the assimilable substrate is labelled with ^{14}C, it is noted that not all of the evolved CO_2 is radio-active. This 'priming effect' (Hallam and Bartholomew, 1953; Barrow, 1960; Clark, 1967) on native organic matter breakdown is attributed by Burges (1965) to a requirement for an accessory carbon and energy source for NADPH synthesis in humus degrading micro-organisms; in the absence of the accessory substrate, little humus breakdown is thought to take place. A similar effect in which glucose stimulated vanillin oxidation has also been described (Kunc, 1971). Some evolutionary significance could be attached to this phenomenon in that it represents a mechanism for the permanent maintenance of a complex of substrates potentially capable of serving a wide range of micro-organisms of both the autochthonous and zymogenous variety. Thus it would appear that many of the substrates reaching micro-organisms in soil are very much second-hand. The privileged position of

rhizosphere micro-organisms in this respect has already been noted (p. 13). It is not surprising therefore to find on microscopic examination of soil that micro-organisms tend very much to be concentrated around recognisably plant derived material, micro-arthropod faeces or other dead or dying micro-organisms.

(a) Empirical studies on real soils

A widely scattered collection of papers purporting to discuss the community dynamics of soil micro-organisms may be found in the appropriate journals. On examination, it is obvious that few of the authors use the term 'dynamics' in the same sense. For the purpose of this study, community dynamics is defined as the changes which take place in growth-correlated population or community parameters over a period of time. Growth-correlated parameters include cell numbers, biomass and metabolic activity; the choice of an appropriate parameter is discussed later (p. 53 and 139). In these terms, the production of quasi-quantitative floristic lists of micro-organisms cannot be considered as a study of microbial dynamics. Such lists, resulting often from cultural experiments involving a single sampling have been popular in the past, but in studies of dynamics are only of value if sampling is frequent and regular. Neither does a simple listing of the types of between-microbe interactions known to occur in soil constitute a study of dynamics (e.g. Clark, 1969; Stotzky, 1973) although such information can provide a very useful background.

Strict application of the definition reduces the number of reports to be discussed to very small proportions. The earliest and most important experiments were carried out at Rothamsted (Crump, 1920; Cutler and Crump, 1920; Cutler, Crump and Sandon, 1922). This monumental series of experiments has made the greatest single contribution to the subject of soil microbial community dynamics. Unfortunately, as in the case of Gause's equally important theoretical study of microbial interactions,

microbiologists have apparently been extremely reluctant to extend this line of research. Crump's (1920) first set of experiments involved sampling at weekly intervals; protozoan and bacterial numbers were estimated by cultural means while temperature, rainfall and soil moisture content were also recorded. Large fluctuations in the numbers of different kinds of soil micro-organisms were observed and despite the generally unsatisfactory nature of the techniques used (i.e. the selectivity of plate-counts for bacteria and the lack of differentiation between cystic and trophic protozoa) several important conclusions were reached. In the two soils studied, the protozoan population was largely confined to the top 6 inches; a comparatively large number of flagellates, amoebae and thecamoebae were present in the trophic condition and a definite inverse relationship between bacterial and amoeba numbers existed. No effect of moisture content, temperature or rainfall was noted on the amoebae, a conclusion which in the short term is to be expected (p. 11). These conclusions were validated when the experiments were extended to include daily sampling (Cutler and Crump, 1920). In this paper, differentiation was made between trophic and cystic protozoa. A daily fluctuation in the numbers of trophic forms of three flagellates Oicomonas, Cercomonas and Bodo sp. was also noted.

The experiments were then continued, protozoa and bacteria being estimated daily for a year (Cutler, Crump and Sandon, 1922). Six kinds of protozoa occurred sufficiently frequently to permit statistical analysis; these were differentiated into cystic and trophic forms while the bacteria were considered as a group on the basis of plate-counts. From this vast amount of invaluable

data, only qualitative conclusions may be drawn; this is a result of inadequate techniques and was recognised by Cutler et al. 14-day averages of the daily numbers demonstrated that well marked seasonal variations in the numbers of soil micro-organisms were superimposed on the daily variations. This is illustrated in Figure 4, where the spring and autumn flushes (p. 12) are noticeable in the bacterial and protozoal counts. Similar seasonal flushes in microbial numbers and activity have been noted by other authors(e.g. Skinner, Jones and Mollison, 1952; Latter et al, 1967; Babuik and Paul, 1970). It is obvious that these seasonal changes are not uniformly correlated with soil moisture or temperature changes. The now-expected inverse relationship between bacteria and amoeba was found in 86% of the observations (Fig. 6). It was found that different kinds of organism displayed different types of fluctuation in numbers. Although there was a slight tendency for the various species of flagellate to fluctuate together from day to day this was not found between two species of amoeba although it was found that both were inversely correlated with bacteria. Perhaps the most obvious feature of the fluctuations reported by Cutler et al is their (non-random) variability. In one case only, a regular two day periodicity was noted (for Oicomonas termo, Fig. 5). Although of regular frequency, the amplitude of this oscillation appears to vary widely between two sets of extremes. Some modification of the classical predator/prey relationship (Gause, 1934) may explain the amoeba/bacteria relationship, but this possibility was apparently not explored by Cutler et al. Periodicity of other protozoa was

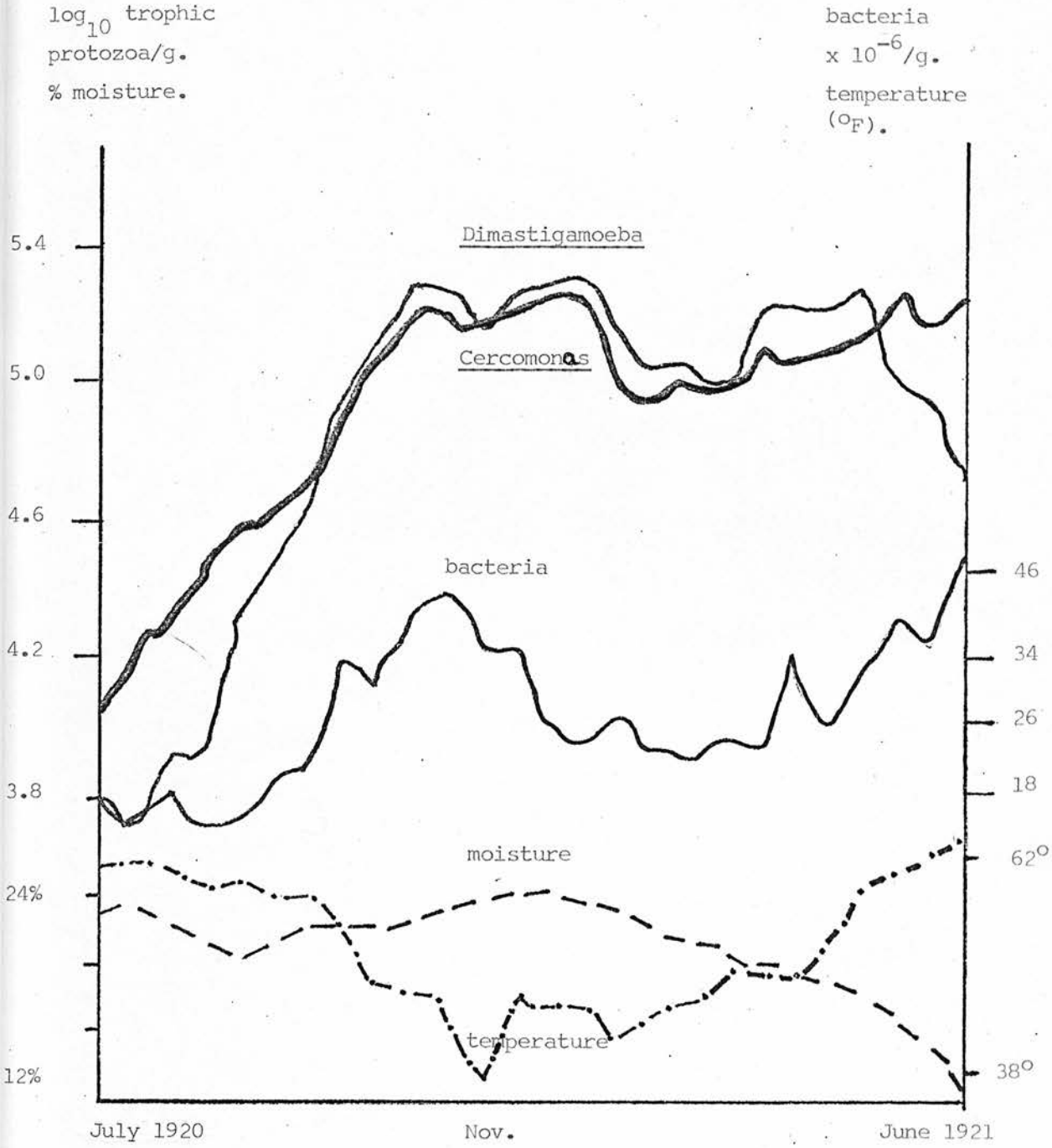


Figure 4. Seasonal variation in soil bacteria and protozoa. (Simplified from Cutler et al, 1922)

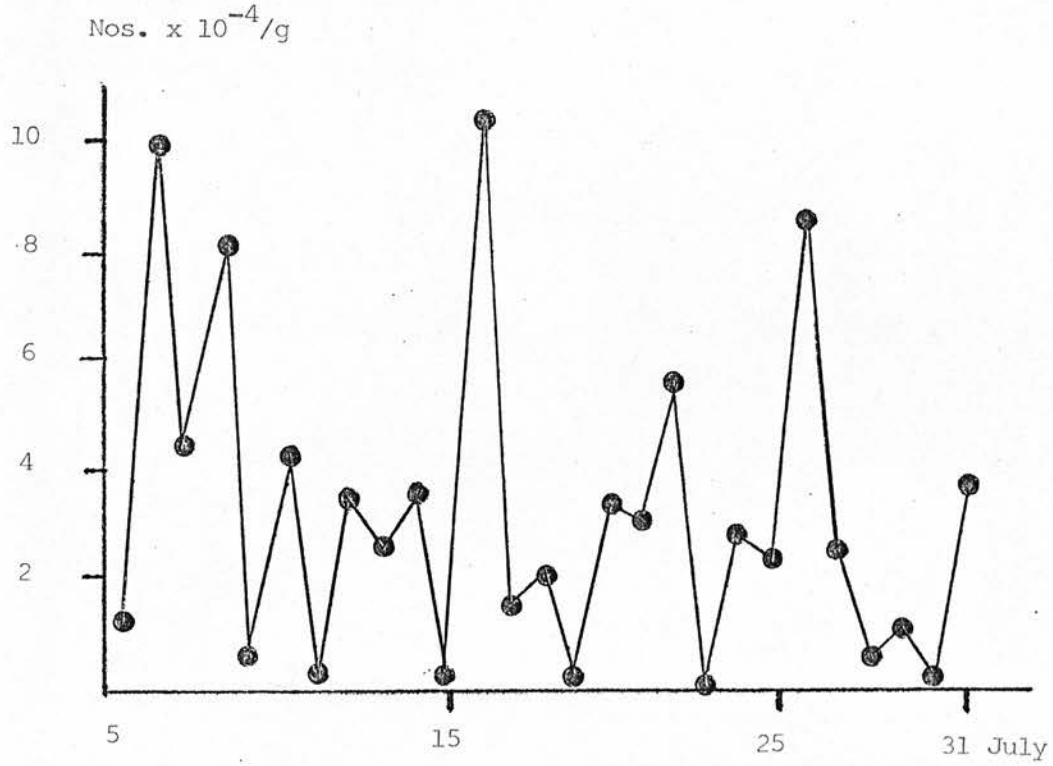


Figure 5. Daily variation in Oicomonas termo (Cutler et al, 1922)

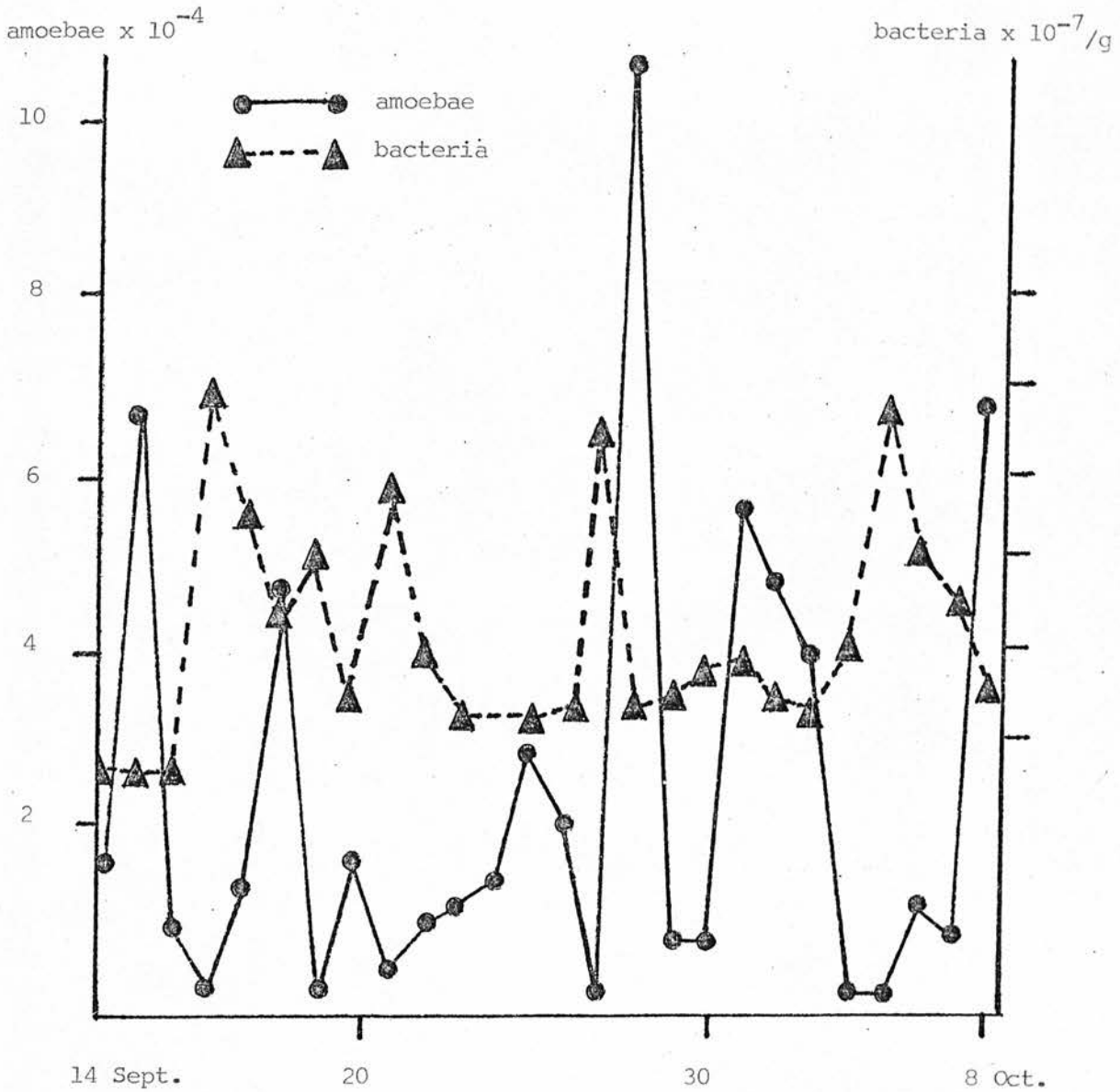


Figure 6. Oscillations of bacteria and Dimastigamoeba. (Cutler et al, 1922).

regarded by these authors as being based on a 'physiological clock' type of mechanism. While this may be true for the holophytic or partially holozoic flagellates no evidence for or against the hypothesis has been derived from studies of soil micro-organisms *in situ*.

On the basis of these studies by the early Rothamsted workers a general picture of soil microbial community dynamics emerges: The highly periodic nature of the system would appear to be its most important attribute in terms of community function (p. 1). The rate and extent of thermogenesis and mineralisation will depend on the number of cycles of population growth and senescence (or death) which takes place in a given time interval. The short term fluctuations appear to be governed from within the community i.e. depend upon community structure in physiological and nutritional terms. Upon these are superimposed seasonal effects of an obscure nature. It would appear that a response in community dynamics to changes in climatic factors can only be expected when such factors are effective for very extended periods i.e. sudden cold snaps, heat waves or downpours can be expected to have only a local transitory effect on community dynamics.

The work of Cutler *et al* was later extended at Rothamsted by Taylor (1936) who as well as recording daily variations in bacterial numbers, demonstrated the existence of much higher frequency oscillations by means of a two hour sampling interval and a variation on the total microscopic count procedure (Thornton and Gray, 1934). Taylor showed statistically significant variation in counts in a 48 hour experimental period. The

most significant finding of this series of experiments was the statistical superiority of the total cell count over the plate-count as a means of detecting cell number variations. Taylor's experiments into the mechanisms of the fluctuations are also of interest, since he demonstrated by soil inoculation that bacteria can oscillate in numbers under steady environmental conditions and in the absence of protozoa.

Until very recently, the lessons of the early workers in soil microbial community dynamics had been largely ignored. Thus Jakubczyk (1970) presented plate-count data of very limited value in a study of the microbial dynamics of four meadow sites. Sampling was at monthly intervals and the whole year was not covered; this may well be the reason that a single seasonal peak in bacterial numbers was observed. Kozlov (1967) compared three sites with varying sampling frequency; in the light of the findings of Cutler et al this procedure appears to be very suspect. From a study of the variation in plate-count over a three month period involving minimally 4 and maximally 8 samplings, Kozlov concluded that the number of bacteria was well correlated with soil moisture content and was "chiefly determined by it". His graphs however show almost the exact opposite. This anomaly is not resolved in the paper or the symposium discussion which follows it.

Recent work by a variety of Russian authors is now compensating for the long period of disinterest in this branch of soil microbiology. Much of the published work has yet to be translated into English, consequently it can only be discussed tentatively

on the basis of available English abstracts. According to Aristovskaya (1972) significant increases and decreases in bacterial numbers take place every 2-4 days in a number of different soils in the U.S.S.R. Seasonal bacterial production figures calculated on the basis of such fluctuations in numbers indicate that a hitherto unconsidered source of nutrients must be responsible for a significant proportion of the bacterial production. Aristovskaya (loc. cit.) suggests that root secretions, algal production and bacterial CO_2 fixation (heterotrophic as well as autotrophic) may be of greater importance in this context than is generally realised. According to Shtina (1972) algal biomass may range from 20-500 kg/ha in the surface (0-2 cm) layers of cultivated soils while in virgin soils it may reach 4 t/ha. The surface layer of algae is also considered important, sometimes reaching 750 kg/ha in cultivated soils. Regular periodic oscillations in bacterial numbers have been noted by several workers (Parinkina, 1972; Schapova, 1972; Zykina, 1972) who used daily sampling and a microscopic cell count procedure. Comparable biomass, production and generation time data were obtained by these authors.

The most complete report of this type of approach which is generally available is that of Parinkina (1973). This author described oscillations in bacterial numbers in several different soils of the Taĭmyr (tundra) region. Values for production, generation times and the number of generations per month are presented, but perhaps the most interesting conclusions to be drawn from this work are of a qualitative nature. Some of Parinkina's graphs are reproduced in Figure 7. In Fig. 7a, the

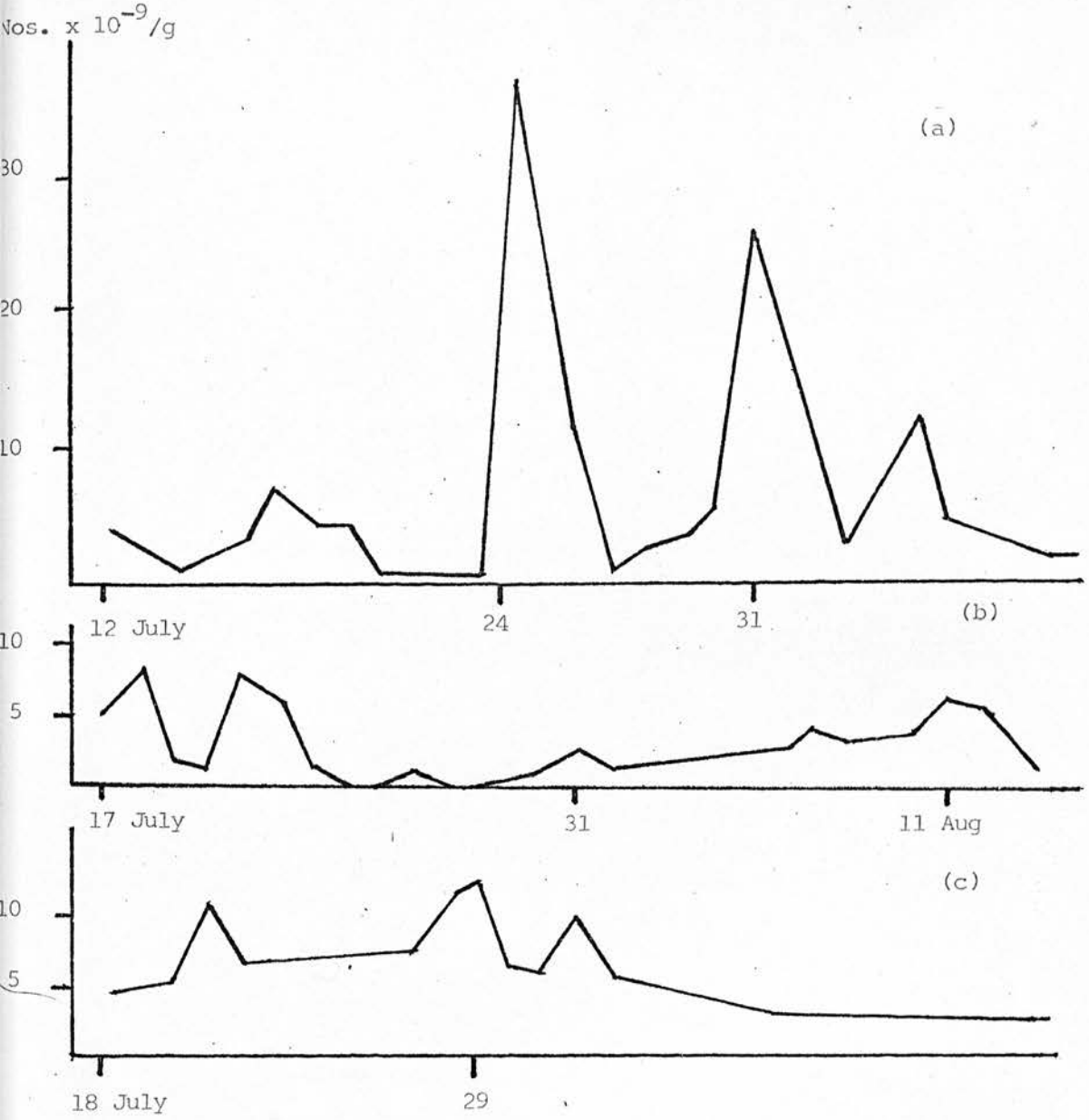


Figure 7. Fluctuations of bacterial numbers in tundra soils. (After Parinkina, 1973).
(a) sandy loam of the crustal layer, frost boil tundra
(b) $A_o A_1$ horizon of main surface, frost boil tundra
(c) polygonal bog peat.

familiar variable amplitude/relatively constant frequency oscillations are shown (cf. Figs. 5,6); in Figs. 7b,c however there appears to be a qualitatively different kind of picture. A background trend rising in Fig. 7b and arching shallowly in Fig. 7c is superimposed upon higher frequency oscillations around this altered baseline. This is not referred to by Parinkina. A possible explanation of this behaviour is that components of the bacterial community are behaving at least partly independently so that at least two types of bacterial production are taking place simultaneously. Classically, these could be referred to autochthonous and zymogenous groups of micro-organisms. The fact that the soil horizons in which this phenomenon takes place have lower standing crops of bacteria than the crustal layer (Fig. 7a) would tend to support this assumption. In these relatively poorer environments it may be argued that a larger proportion of the microflora may be displaying slow autochthonous activity, whereas the more favourable environment could support a more rapid zymogenous type of response. Consequently in the more favourable site, a greater proportion of the bacterial community may be made up of more rapidly reacting bacteria. This reasoning is substantiated by the finding of a qualitatively similar kind of oscillation to that shown in Fig. 7a in the A horizon of a tundrasod soil supporting a similar range of standing crop of bacteria to the frost boil tundra crustal layer (Fig. 7a) (Parinkina, loc.cit.).

Apart from the mystery which surrounds the source of energy and carbon tapped by the micro-organisms whose oscillations are

reported, the problem of synchrony is important. If oscillations of the type discussed above are real, the data imply that the whole sampling site in each case is oscillating in synchrony. Otherwise, the variations in count from soil sample replicates from adjacent sites would tend to cancel out. This conclusion requires a global effect to be responsible for the oscillations; in the case of the tundra soils, this could be supply of nutrients after freezing. In temperate soils, drying and remoistening appears to be a more likely candidate.

The definition of microbial community dynamics (p.33) allows inclusion of temporal changes in the qualitative nature of the microflora in the subject. Very little experimental data is available however; some work has been done on the seasonal succession of fungi (Toenjes et al, 1957; Miller and Boothroyd, 1962) but the frequency of sampling and general applicability of the results is low. Observations of seasonal variations in microbial activity (which also fall within the definition) are slightly more satisfactory. The interpretation of seasonally varying enzyme activity in soil is complicated by the influence of plant roots and varies from soil to soil. Thus Gyllenberg (1957) found that the rhizosphere microflora of oats was nutritionally constant throughout the growing season but that the microflora of non-rhizosphere soil gradually tended towards that of the rhizosphere as the season drew to an end. Effectively this may be interpreted as an extension of the rhizosphere and may be correlated with the importance assessed to root secretions by Aristovskaya (1972). The relative contributions by roots and micro-organisms to soil enzyme pools are difficult to determine.

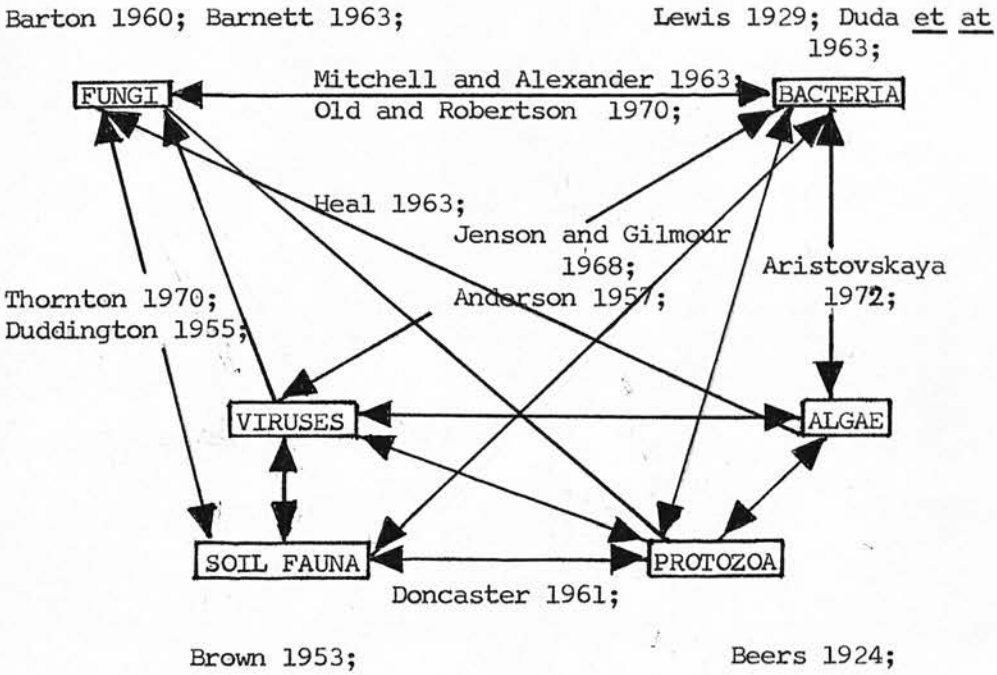
However, Ragoutis (1967) did find correlation between microbial population levels, respiration intensity and enzyme activities in forest soils; he also observed a spring population and activity flush although respiration intensity was maximal in summer. After a decline towards the end of summer, an autumn flush in biological activity was recorded. A similar spring flush, summer decline, autumn flush sequence was noted by Galstyan (1965) in invertase, polyphenol-oxidase, urease, β-glucosidase and amylase activity in a chernozem, a chestnut-carbonate and a brown non-carbonate soil. In some extreme environments e.g. sodium-solonchak soils, extracellular enzyme activity is inhibited by high alkalinity and soluble salt concentration. In late summer, Galstyan (1964) failed to detect any enzyme activity in this type of soil. The lack of information as to qualitative seasonal changes in the soil microflora is unfortunate; however, differing seasonal maxima for proteolytic and cellulolytic activity have been noted by Zaguralskaya, Yegorova and Smantzev (1972) in Karelian swamp peats. Maximum proteolytic activity was observed in early summer and cellulose decomposition was most intense in the autumn. An autumn maximum of cellulolysis was also noted by Yershov (1972) in Karelian meadow soils; in the winter/spring period, the intensity of cellulolysis dropped sharply. It might be expected that seasonal changes in soil composition would accompany changes in microbial growth; these could only be expected to be minor and little experimental data appears to be available. Ivanov (1970) has however detected seasonal changes in the composition of soil solutions in the western Taĭmȳr.

(b) Between-microbe interactions occurring in soil

It has been tentatively noted in the preceding discussion that short-term oscillations in microbial numbers are probably products of between-microbe interactions rather than of variations in environmental parameters. Consequently it is of interest to consider the range of types of interaction which may occur in soil microbial communities. In itself, this subject is vast, consequently it cannot be discussed in any depth and must simply be summarised briefly. Any one taxonomic group of micro-organisms may simultaneously interact with a large number of different taxonomic types, thus to consider a bacterial/bacterial, bacterial/fungal or protozoan/fungal interaction represents a concentration on a part (of unknown size) of the problem to the exclusion of the (unknown) remainder. Thus the simplest units in this type of system are the interactions themselves. These may be stimulatory or inhibitory. In broad terms the mechanisms of stimulation are: provision of nutrients, production of non-nutrient stimulatory metabolites and physical protection. Mechanisms of inhibition include lysis, ingestion and production of inhibitory metabolites (of varying specificity). The subject of interactions between soil micro-organisms has recently been extensively reviewed and discussed by a number of authors (Clark, 1965, 1967b, 1969; Stotzky, 1973). These works are copiously referenced and repetition is unnecessary.

The scheme outlined below represents directional interaction (by the mechanisms outlined above) between different kinds of soil organisms. As it stands, it represents best the possible range of inhibitory interactions. With the removal of the viral compartment, the same scheme serves equally well for stimulatory interactions.

It may not be an exaggeration to say that all conceivable kinds of between-microbe interactions may occur in soil microbial communities. These may be simple, involving say two types of organism, or may comprise a complex web of different organisms linked in such a way that disturbance of one member has widespread and distant repercussions (e.g. Burbanck and Ewan, 1960).



Scheme of interactions between soil micro-organisms; arrows represent direction of stimulation or inhibition.

(c) Theoretical consideration of between-microbe interactions

Because of the heterogeneous nature of soil (Hammond et al, 1958; Gallagher and Herlihy, 1963; Ball and Williams, 1971) and its contained microbial communities, its high variability within and between types of environment and the lack of generally applicable mathematical theory, little or no predictive mathematical modelling has been directly applied to soil microbial community dynamics. At the opposite extreme insufficient field data exist to allow even empirical mathematical description of the complex phenomena which are known to occur. The mathematical description of between-microbe interactions in controlled homogeneous systems is in its infancy (Contois and Yango, 1964; Stutzenberger and Bennet, 1965; Ramkrishna et al, 1966; Bungay, 1968; Meers and Tempest, 1968; Williams, 1973) but a consideration of the types of approach adopted and of the results obtained is necessary to maintain the correct perspective in the investigation of complex soil systems. One particularly relevant finding of many laboratory and theoretical studies on interacting micro-organisms is that the outcome of interaction very often results in oscillatory changes in the numbers of the interacting organisms (e.g. Bungay, 1968). Such oscillations may be convergent towards or divergent from an equilibrium which itself may be oscillatory.

The basic approach adopted in mathematical treatment of simple interactions (i.e. 2 or a small number of interacting species) has been to utilise functions describing the behaviour of the components under axenic conditions where possible. These functions are then modified by the addition of terms which take into

consideration the effect of the other member(s) of the mixed culture. The basic shape of population growth curves is familiar to all microbiologists; its mathematical description is not however so cut and dried as may be expected. The first and best known model for the sigmoid growth curve was the logistic (or Verhulst-Pearl) equation:

$$N_t = K/(1 + e^{a-bt}) \qquad (3)$$

(N = number of individuals present; 'K' is the number present at habitat saturation; 'a' and 'b' are constants related to the steepness and height of the curve; t is time).

A more readily assimilable form of the equation is:

$$dN/dt = rN.(K-N)/K \qquad (4)$$

(dN/dt = instantaneous rate of increase; 'r' = innate capacity for increase).

When $N \ll K$, this reduces to

$$dN/dt = rN \qquad (5)$$

i.e. a simple logarithmic rate of increase and the familiar equation for exponential growth. The logistic equation (3) has been applied by Lotka (1924) to the colonial growth of Bacillus dendroides (a variant of B.mycoides, Gibson, 1971) on the basis of data provided by Thornton (1922). The theoretical curve provided an excellent fit of the experimental data. On the basis of the logistic equation, Lotka (loc.cit.) and Volterra (1926, 1931) derived systems of equations describing changes in population density of interacting species. These have come to be

known as the Lotka-Volterra equations and a simplified introduction to their application in studies of interacting organisms with non-separate generations is provided by Maynard-Smith (1968). The first and most significant application of this theory to micro-organisms was presented in an eminently lucid book by Gause (1934). He studied a variety of interactions including competition and predator/prey relationships using yeasts and protozoa as experimental organisms. As predicted by the equations Gause found oscillations in numbers in predator/prey relationships. This is simply understood in terms of the predator reducing prey concentration to a point where the predator population can no longer be maintained. As a result of its decline the environment becomes more favourable to the prey which multiplies and the cycle is repeated until the prey food supply becomes limiting. In situations where the efficiency of prey capture is high, both populations rapidly become extinct as a result of the removal of the last prey organism at an early stage.

Although a number of interesting conclusions may be drawn from such analyses, it is wise to heed Slobodkin's (1961) warning."... ...by suitable definition of terms, alteration of signs of coefficients and perhaps the addition of one or two coefficients to the equation, we can convince ourselves that we are developing a theory of ecology while we watch the empirical ecological world slowly disappear into the limbo of natural history. Therefore, rather than expand this two species model, it is advisable to return to the initial assumptions underlying our original sigmoid growth equation for a single species."

More recently a renewed interest in the analysis of microbial

interactions has grown up, primarily in the fields of water, sewage and industrial microbiology and chemical engineering. The preferred experimental tool in most modern studies appears to be the chemostat. As well as numerous advantages, this also confers on the investigator a need to modify the analysis. The continuous overflow of the chemostat represents a non-specific rarefaction on the interacting organisms. Analogous effects of non-specific predation on interacting organisms can produce interesting repercussions (Slobodkin, 1961). Thus the outcome of competition may be reversed by a non-selective predator. Two species may continue to exist in a space in which only one could exist without predation, or conversely predation may upset an equilibrium between species so that one will disappear in competition. In a two-species interaction in which the outcome is dependent on initial concentration of organisms, predation can eliminate the possibility that the species with the lower innate reproductive rate will ever win the competition. This is directly analogous to the classical chemostat enrichment of micro-organisms with maximal reproductive rates.

The basic mathematics of chemostat cultures is well known (Monod, 1942; Herbert, Ellsworth and Telling, 1956). In ideal experimental conditions, mass balance considerations give rise to the equations:

$$dx/dt = \mu x - Dx \qquad (6)$$

$$dS/dt = DS_o - DS - \mu x/Y \qquad (7)$$

(x = mass concentration of organisms; D = dilution rate; μ = specific growth rate; S = substrate concentration (S_o in feed); Y = yield constant.)

The specific growth rate is a function of the limiting substrate concentration, described empirically by the so-called Monod saturation equation:

$$\mu = \mu_{max} \cdot (S/(K+S)) \qquad (8)$$

(μ_{max} is the maximum growth rate (innate reproductive capacity) and K is a constant).

Application of the Lotka-Volterra approach to continuous culture of predator/prey systems gives the equations (Bungay, 1968):

$$dH/dt = \mu H - DH - k_1 HP \qquad (9)$$

$$dP/dt = k_2 HP - DP \qquad (10)$$

(H = concentration of prey (hosts); P = concentration of predators (parasites); μ = specific growth rate of prey; k_1 = killing efficiency based on encounters; k_2 = predator growth rate constant).

This model is equivalent to a predator growth rate linearly related to its food concentration (Gause, 1934). This is an unreal assumption since the normal type of saturation relationship is to be expected as found by Proper and Garver (1966) for the ciliate Colpoda steinii feeding on Escherichia coli. Consequently Monod-type saturation kinetics for predator specific growth rate and prey concentration were included by Bungay (loc.cit.) in a basic treatment of chemostat studies of predator/prey relationships.

It is interesting to note that in a recent study (Williams, 1973) the description of the relationship between growth rate and nutrient concentration by Monod saturation kinetics was considered inadequate. At low turnover rates, metabolic losses cause a decline in biomass

and numbers and extinction of the population at a threshold or 'compensation point' where metabolic losses are greater than growth at very low limiting nutrient concentrations. A convenient exit from this morass of empirical mathematics can be found in the statement of Bungay (loc.cit.) that the Lotka-Volterra model with its false assumption about predator growth rate fits experimental data about as well as the model with the Monod relationship. Williams (loc.cit.) also noted that equally satisfactory results could be obtained from mathematical theories embodying the logistic equation, Monod saturation kinetics or compensation kinetics. Consequently it should be emphasised that a good fit to the experimental data, while essential, does not prove the validity of the mathematical model.

These and other complexities of the mathematical approach have led to the use of computer models as aids in understanding population interactions, in assessing the usefulness of equations and in predicting the most fruitful lines of research. In this way functional predictive models can be obtained and in the case of analogue computer models, the effect of varying constants and equations can be watched directly on an oscilloscope screen. The use of such models in studying microbial interactions is outlined by Bungay (1968). An essentially similar approach can be adopted by using digital computer simulation techniques, profitable use of which is described by Curds (1971) in a study of predator/prey relationships involving Tetrahymena pyriformis and Klebsiella aerogenes.

3 DIFFICULTIES IN STUDYING MICROBIAL COMMUNITY DYNAMICS IN SOIL

Despite the fact that the foundations of quantitative population dynamics were laid in Gause's experiments with micro-organisms, microbiologists have largely ignored the subject until quite recently. Consequently before considering the problems in studying microbial dynamics in soil, it is necessary to turn to a text on general population dynamics for a complete definition of the subject and its ramifications. The introductory treatment by Solomon (1971) is adequate for this purpose, illustrating the state of ignorance in which soil microbiologists must approach the subject. Solomon defines the subject as being concerned with a series of inter-related topics:

(1) the number of individuals making up the population of particular species in specified places;

(2) the changes in these numbers that are observed over a period and the differences in abundance in different places;

(3) the rates of reproduction and mortality and of gains or losses by dispersal which together account for the changes in numbers;

(4) the operation, singly and in combination of the various factors influencing these processes of gain or loss - the effects of weather and other environmental factors, the effects of natural enemies and disease, and the effects of food shortage, crowding etc.;

(5) the processes within the population as it reacts to this complex of influences both internal and external; and

(6) the relative importance and the interaction of (i) processes that tend to regulate abundance, and (ii) those that simply change it.

These topics can be simply translated into microbial terms on the basis of information supplied in the preceding parts of this discussion. Although the subject thus defined assumes immense proportions, the basic technology of soil microbiology is such that only small portions of the subject can be investigated at present. The remainder is open to more or less inspired speculation. The term microbial community dynamics has been used extensively in this study; this is of necessity since at present no techniques exist for studying the whole community and its assemblage of populations simultaneously. It is also unknown whether there is any real significance in applying the monospecific population concept to soil microbiology. A species defined in terms of taxonomic affinities will certainly make up a statistical population in soil, but in functional terms the ecological population may well be made up of a number of taxonomic species (see also p. 178). Hence the term community is retained, implicit in this usage being the possible existence of more or less complex functional sub-units (populations) within it. The complexity of the soil environment and its biotic components has been stressed sufficiently for the general conceptual difficulties of approach to be readily apparent. Practically, progress is restricted by a lack of adequate techniques and background information.

Some knowledge of the qualitative nature of soil microbial communities may be considered a prerequisite for a study of their dynamics. No adequate means of providing this information exists at present, dependent as it is upon:

(I) adequate spatial sampling to allow for local soil heterogeneity;

(II) adequate frequency of sampling to allow for the qualitative and quantitative changes which are known to occur in the composition of the community;

(III) extraction and identification of all the kinds of micro-organisms present; and

(IV) extrapolation from the identification to the possible ecological significance of the organisms.

(I) and (II) are feasible with considerable exertion; (III) is frankly impossible at present and is likely to remain so in the foreseeable future. (IV) would be difficult if (III) were possible.

In most ecological investigations, samples taken from an environment are analysed and the analytical data extrapolated to the whole environment; complete sampling of a natural environment is rarely possible. Such a procedure necessitates knowledge of the relationship between sample heterogeneity and environment heterogeneity; both of these factors normally require knowledge of sample size relative to environment size. In quantitative soil microbiology the assessment of environment boundaries is difficult and often arbitrary since in microbial terms the environment size is infinitely smaller than anything which could be satisfactorily sampled. Thus it is necessary to sample probably millions of micro-environments simultaneously. Since no knowledge of the frequency distribution of organisms within the micro-environment can be available before sampling, heterogeneity of samples (in terms of micro-environment distribution) can only be judged on the basis of the total number of micro-organisms per arbitrary unit of some sample parameter

(e.g. weight, organic matter content, internal area etc.). That this frequency distribution will be dependent on sample size is obvious since it is almost inevitable that centres of microbial activity will be non-uniformly distributed. The effect of varying sample size in such a system can be judged from Figure 8. The problem is normally circumvented by taking a comparatively very large soil sample.

In considering environment boundaries it is also of interest to note that a factor considered of tremendous importance by (mainly animal) ecologists is immigration and emigration of organisms; this has received virtually no attention from soil microbiologists.

As noted earlier (p. 50) it is of importance to differentiate between environmental factors which regulate and those which simply alter microbial equilibria. In the light of the early part of this discussion, it is obviously impossible to measure certain simple environmental parameters (e.g. pH) at the site of their effect on micro-organisms. This problem is however conceptual as well as practical since no general knowledge exists of the micro-environmental parameters which are of major importance to the various kinds of micro-organisms which make up the community. Consequently it is very difficult to differentiate the types of effects of different environmental parameters on community equilibria.

As noted above (p. 50) classical population dynamics is concerned with changes in and equilibria of numbers of individuals. In soil microbiology, the relevance of numbers may often be suspect.

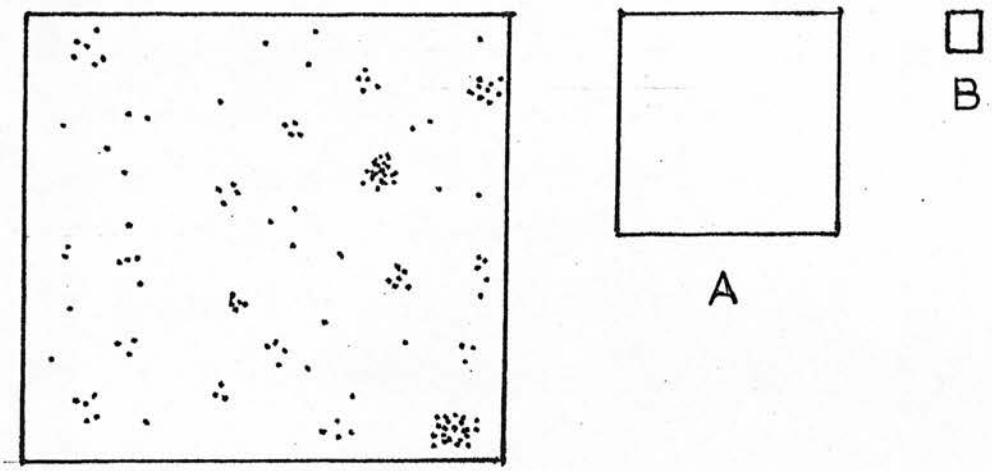


Figure 8. Dependence of percentage frequency on quadrat size. (After Kershaw, 1964) The two quadrats A and B give widely differing frequencies for the same community.

This can be simply demonstrated by a hypothetical example. Consider a soil microbial community made up of only two types of bacteria, both of which are spherical,

Let one of them (A) be 1 μm diameter

Let the other (B) be 2 μm diameter,

now the ratio of any linear dimensions A/B = 1/2, hence the volume ratio A/B = $1^3/2^3$ = 1/8. Assuming identical densities, this is also the weight ratio.

Consider the situation where A and B can use the same substrate (present to excess) with the same efficiency.

Let A have a generation time 1/3 that of B.

If the initial community at time o is made up of equal numbers of A and B, then after time t in which B undergoes one doubling in numbers, A has undergone three doublings. Numerically the ratio A/B at time t is 8/2 i.e. the community is 80% A, 20% B; the weight ratio A/B is 8/16, i.e. in biomass terms, the community is 33.3% A, 66.6% B at time t. The ratio of overall substrate weights consumed by both members of the community is however 7/8 i.e. in time t, B has been responsible for slightly more substrate transformation than A. Similarly, after time 2t the community would be numerically 93.75% A, 6.25% B. In biomass terms it would be 66.6% A, 33.3% B. In the total time 2t, 'A' component would have been responsible for the transformation of 63/24 times as much substrate as 'B' component.

After considerations of this kind, it becomes clear that cell numbers alone (even ratios of cell numbers) are almost without value. Equally, biomass data alone is without value in dynamic studies. It is obviously necessary to have some means of estimating

biomass and activity simultaneously. Since a significant activity parameter must be related to the function of the community as defined earlier (p.1), estimations of population turnover (production) and assimilation efficiency are desirable. A knowledge of so many community parameters is however very much an ideal since technically there is no completely satisfactory method of determining cell numbers or biomass. In the presence of a heterogeneous microflora utilising an unknown range of substrates, knowledge of assimilation efficiency also seems somewhat distant. It is obvious that technical difficulties are immense; since these are discussed in detail later they are not elaborated upon here. Perhaps the crowning difficulty in this subject is the fact that all current techniques for the estimation of numbers or biomass destroy the sample completely. Since as defined, a study of dynamics involves observation of a community over an extended time period, this problem presents its own peculiar difficulties. The importance of these lies in the heterogeneity of soil samples and the distribution of microbial colonies within them.

STATEMENT OF INTENT

The study of microbial community dynamics in soil is confused by the fact that an immeasurably large number of microbe/microbe and microbe/abiotic environment interactions takes place simultaneously. The possibility exists that understanding of this situation could be facilitated if component populations within the community could be considered as functional units (Brock, 1966). It is an empirical fact that when a system is complex enough to require many components, the phenomenon of unitisation occurs (Quastler, 1958). This means that some components of the system become organised in such a way that interacting strongly among themselves, they act as a unit with respect to the remainder of the system. Justification for the adoption of this concept and its translation into microbial terms are discussed in p. 182 et seq.

It is the object of this study to find out whether this unitisation hypothesis can be profitably applied to a study of soil microbial community dynamics. This aim poses a secondary question of whether or not available techniques in soil microbiology are adequate for such an investigation. An appraisal of these two major problems constitutes the work presented in this thesis.

If a steady state soil microflora is considered in the light of the unitisation hypothesis, it is clear that some coupling (however loose) must exist between the units. However, for the efficient internal function of the units it is clear that interaction between components of different units must not be extensive unless one postulates complete tight coupling between units. The likelihood of tight coupling is remote, not only because of the potentially enormous numbers of individuals involved but also because such an arrangement would be inherently inflexible and would tend to limit severely the community's homeostatic capacity.

Thus (theoretically at least), the possibility exists of investigating the behaviour of components of a microbial community. If this were a practical possibility it would simplify enormously the task of investigating the dynamics of soil microbial communities (Brock, 1966). Thus the behaviour of groups of organisms which functioned in concert could be studied; this would be a great advance over mere extrapolation of pure culture data to the soil system.

In order to gain an insight into the functioning of microbial communities, an understanding must be gained of the factors which in combination produce and influence such communities. In a closed system (i.e. one which is capable of functioning only for a limited time since biological efficiency is less than 100%) sequential population interactions may take place, but in the presence of a nutrient environment which is discontinuous in time, the only logical outcome of such activity in this environment is death. Clearly since micro-organisms are currently an integral functioning component of the biosphere, the closed system

environment must be regarded as a transient situation subsidiary in importance to open systems. It should be added that a theoretically completely closed system is insulated from the biosphere; in reality, superinoculation of 'open' and 'closed' systems occurs as a result of the function of microbial dispersion mechanisms.

Open system conditions are potentially capable of supporting steady state communities. However in view of the irregularly intermittent nature of the nutrient input into the soil, this steady state is unlikely to approach the inviolable stability of a chemostat or turbidostat culture. Because the chemical and biological status of a specific soil type is relatively constant in terms of climax primary production over a reasonable time scale (ecologically speaking) it can be assumed that in such circumstances a steady state microbial community is already apparent in terms of function. This situation can evolve provided the rate of change in the environment does not outstrip the rate of community adaptation to accommodate the change. The soil microbial community is likely to exhibit features of both open and closed system situations; sequential population development as a result of sporadic nutrient additions occurs. The underlying steady state is apparent in the reproducibility of a specific response to a specific environmental stimulus, e.g. the annual cycles of decomposition referred to earlier (p.34 and 41). The soil microflora is thus composed of a number of organisms of different genotypes functioning in such a way as to perpetuate the potentialities of the steady state system.

From a consideration of natural selection it is obvious that a

soil microflora must function at the maximum efficiency possible in its specific environment otherwise it would realise its capability of adapting to greater efficiency. The 'specific environment' must be defined in temporal as well as physical terms; clearly a microflora which rapidly and completely mineralises all nutrient materials is not efficient in this sense. On depletion of nutrients, the system would become effectively closed until the next spate of input. Thus an efficient soil microflora must retain a reservoir of nutrients sufficient to maintain that microflora between periods of nutrient influx. It has already been postulated that humic materials can play this role (p. 31). In this light it would appear probable that the functioning of the humus-degrading micro-organisms is a key factor in the maintenance and regulation of the steady state microbial community.

The degree of perfection (in evolutionary terms permanence) of the steady state community must be judged in the context of the extent to which its homeostatic mechanisms allow it to overcome environmental inconstancies. The fact that highly versatile steady state communities capable of existing in balance despite gross environmental changes remain unreported indicates that homeostatic mechanisms in microbial communities are less well developed than the diversification of function and dispersal in individual species. From this line of reasoning, it is obvious that the response of a steady state community to an environmental change which exceeds its homeostatic capacity will be affected in terms of disintegration of community structure. If the now-changed environment remains constant, it is potentially

capable of selecting a fresh steady state community provided it has not changed to the extent that conditions preclude microbial adaptation - in which case the situation has become effectively a closed system and the only equilibrium possible is sterility.

It will be obvious from the above discussion that the function of a community, particularly its capacity to maintain itself in the face of environmental stress, is intimately linked with its structure. The structure of any microbial community must be governed by the interaction of its component phenotypes with one another and with the environment. The role of microbial predators in community homeostasis has been indicated (Mitchell, Yankofsky and Jannasch, 1967; Mitchell and Wirsen, 1968; Mitchell, 1971). In this context, the rejection of alien organisms (Anscombe and Singh, 1948; Klein and Casida, 1967a; Szabo et al, 1968) is a phenomenon well utilised in some methods of sewage disposal. While community structure is obviously based on interactions between microbial phenotypes, homeostatic capacity must depend to a large extent on genotypic capabilities.

It has been necessary to establish the somewhat nebulous notions of the form of microbial communities outlined above, since combined with the technical difficulties, enumerated earlier (p. 57) these factors largely dictate the experimental approach. The use of systems analysis is currently very popular in the study of soil microbiology (see for example Rosswall, 1973). This is essentially the application of standard scientific method in a highly formalised manner. The formalisation is necessitated by the complexity of the subject matter; the long term object of the procedure is to arrive at a mathematical description and simulation of the system

under study. In simpler models, the simulation may consist of a few equations whose coefficients may be calculated directly by the investigator. Such a model is presented by Williams for the steady state of growth of a single microbial species in a study already referred to (p.43). More usually in the study of a natural system, the simulation is complex, involving a large number of variables and equations. Such models are normally fed into a computer in such a way that specific parameters may be altered at will and the effects of this manipulation be predicted in terms of the behaviour of the whole system. The application of such an approach to studies on decomposition in soil is described by Bunnell (1973). Use may be made of this type of approach at any convenient level of organisation within a system. Thus it is possible to obtain functional models of whole ecosystems with respect to say energy transformation, or models concerned solely with carbon and nitrogen transformations and turnover in soil. The latter case is exemplified by the work of McGill, Paul, Shields and Lowe, (1973).

Logic demands that as system simulation advances through conceptual models, verbal descriptions, mathematical descriptions and finally computer models the results and ramifications of each stage in the analytical process be referred back to the real world. This continued comparison with the real world rightly receives great emphasis in the systems analytical approach. It may readily be realised however that application of such theory to an ecological problem takes the form of a project involving a number of specialist investigators, a relatively large number of investigator-years of work and a not inconsiderable budget. Consequently the executor of a study like the present one cannot hope to take an ecological study all the way to its logical conclusion. In such a situation

it is the responsibility of the investigator to lay the foundations of an approach which can be taken up by later workers. Accepting the very limited capabilities of one worker in such a study, it is also important that methods and concepts which are used should not be immutable ones. In other words, methods and ideas should be sufficiently versatile and open-ended to allow for refinement at some time in the future when the need for this will as a matter of certainty become obvious. Thus the analogy between the formalised stages of systems analysis and the stages of any well planned piece of experimental work becomes apparent.

EXPERIMENTAL WORK

SECTION A:

The use of genetically labelled bacteria in microbial community modelling

A(1) (a) Theory - the mathematical model

Determination of microbial numbers is a major methodological problem in quantitative soil microbiology. Microscopic examination of soil gives estimates of ca. 10^9/g but does not differentiate living from dead cells. Viable counts achieved culturally give estimates of 10^6-10^7/g. It is readily demonstrated that viable counts are inaccurate (p.98). At present it is impossible to measure the total number of living bacteria in soil.

By assuming that exponential phases occur during the growth of bacterial populations in soil, a mathematical model incorporating total microscopic and viable count data has been derived. This model estimates the total number of actively dividing bacteria in soil.

Consider a simple community of soil bacteria composed of two populations 'a' and 'V'. Consider that 'V' can be measured by the viable count technique and that 'a' fails to grow on culture media. The only way in which an indication of variation in 'a' may be gained is by a direct microscopic count. At any time, the microscopic count

$$T = V + a + D. \qquad (11)$$

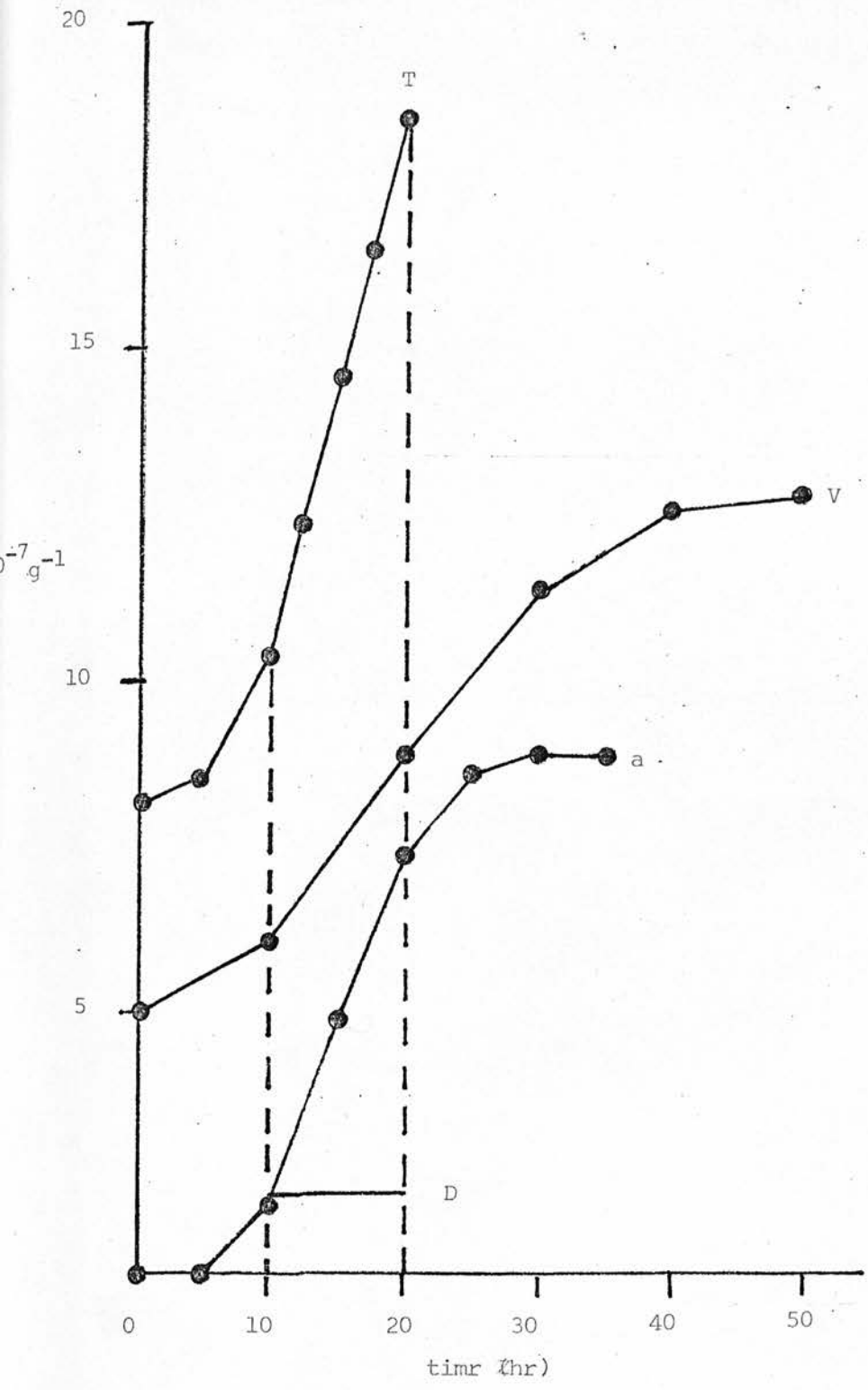


Figure 9. Logistic plot of T, and V after a growth-promoting stimulus at time 0.

where D is the number of dead cells.

During exponential growth, tabulated values of T_t, V_t and t (hr) are obtainable; assuming no dead cells to be produced in the exponential phase:

$$dD/dt = 0 \qquad (12)$$

Now,
$$T_t - V_t = a_t + D. \qquad (11)$$

let $Y = (T_t - V_t)$ then,

$$dY/dt = d(a_t + D)/dt.$$

thus,
$$dY/dt = da_t/dt + dD/dt \qquad (13)$$

substituting from (12),

$$dY/dt = da_t/dt \qquad (14)$$

During exponential growth,

$$\ln a_t = \ln a_o + \mu t$$

(a_o is the number of cells in population 'a' at the start of exponential growth.)

thus
$$a_t = a_o e^{\mu t} \qquad (15)$$

(μ = specific growth rate constant of population 'a').

From (14),

$$dY/dt = da_o e^{\mu t}/dt.$$

Differentiating R.H.S. w.r.t time,

$$dY/dt = \mu a_o e^{\mu t}$$

thus
$$\ln dY/dt = \ln(\mu a_o e^{\mu t})$$

and
$$\ln dY/dt = \ln \mu a_o + \mu t \qquad (16)$$

If a graph of $\ln(dY/dt)$ vs t is plotted, a straight line of gradient μ and intercept μa_o is obtained. Hence a_o may be estimated and D obtained by difference over the range of exponential growth where T and V overlap.
The specific growth rate constant μ defines the number of e-fold increases in 'a' per unit time. The more assimilable growth rate $1/g$ defining the number of twofold increases per unit time is related to μ by the standard identity $1/g = \mu/\ln 2$, whence the population mean doubling time is the reciprocal of this figure. Thus the total number of dividing cells (a + V) may be estimated.

The applicability of this procedure for estimating a + V may be guaged by considering a theoretical example where logistic population growth cycles of T and V (calculated from assumed values of a, V and D) are used as experimental data. The chosen values were derived from logistic curves by the method of Gause (1934). The logistic equations and experimental conditions for each component are:

for a, $N_t = K/(1 + e^{3.109 - 0.33t})$

$K_a = 9 \times 10^7/g$; $N_{o,a} = 1 \times 10^7/g$.

for V, $N_t = K/(1 + e^{0.944 - 0.097t})$

$K_V = 12.85 \times 10^7/g$; $N_{o,V} = 5 \times 10^7/g$.

$D = 10\% (V + a)$.

Since the model applies only to exponentially increasing populations, $\log_{10}$ transformations of the logistic data were plotted (Figure 10). It should be noted that strict adherence to the logistic equation does not define a truly exponential phase since growth is restricted throughout the cycle (equations 4 and 5). Consequently some smoothing of Figure 10 was carried out. The period of simultaneous exponential increase in a and V in Fig. 10 was defined and T calculated. Y was plotted against t (Figure 11) and in view of the limited data, mean dY/dt values were calculated (i.e. $(dY/dt)_{t2} = (Y_{t3} - Y_{t1})/(t_3 - t_1)$ and $t_2 = (t_3 - t_1)/2$.

ln dY/dt vs t was plotted in Figure 12. The slope (μ) of this line was determined as 0.165 whereupon a_o (at t = 8 hr) was estimated as 1.59×10^7 /g. The real value of (V + a) at 8hr was 7.11×10^7 /g (Figure 10); the calculated value of (1.59 + 5.7) = 7.29×10^7 /g is a good approximation. D was estimated by difference as 0.42×10^7 /g (in reality 0.6×10^7 /g).

A (1) (b) Theory - the physical model

Application of the mathematical model to soil may be restricted by (1) problems of obtaining accurate estimates of T and V, (2) interaction of populations V and 'a' and (3) the possibly large numerical differences between T, V and a. To test the effect of these factors a physical model

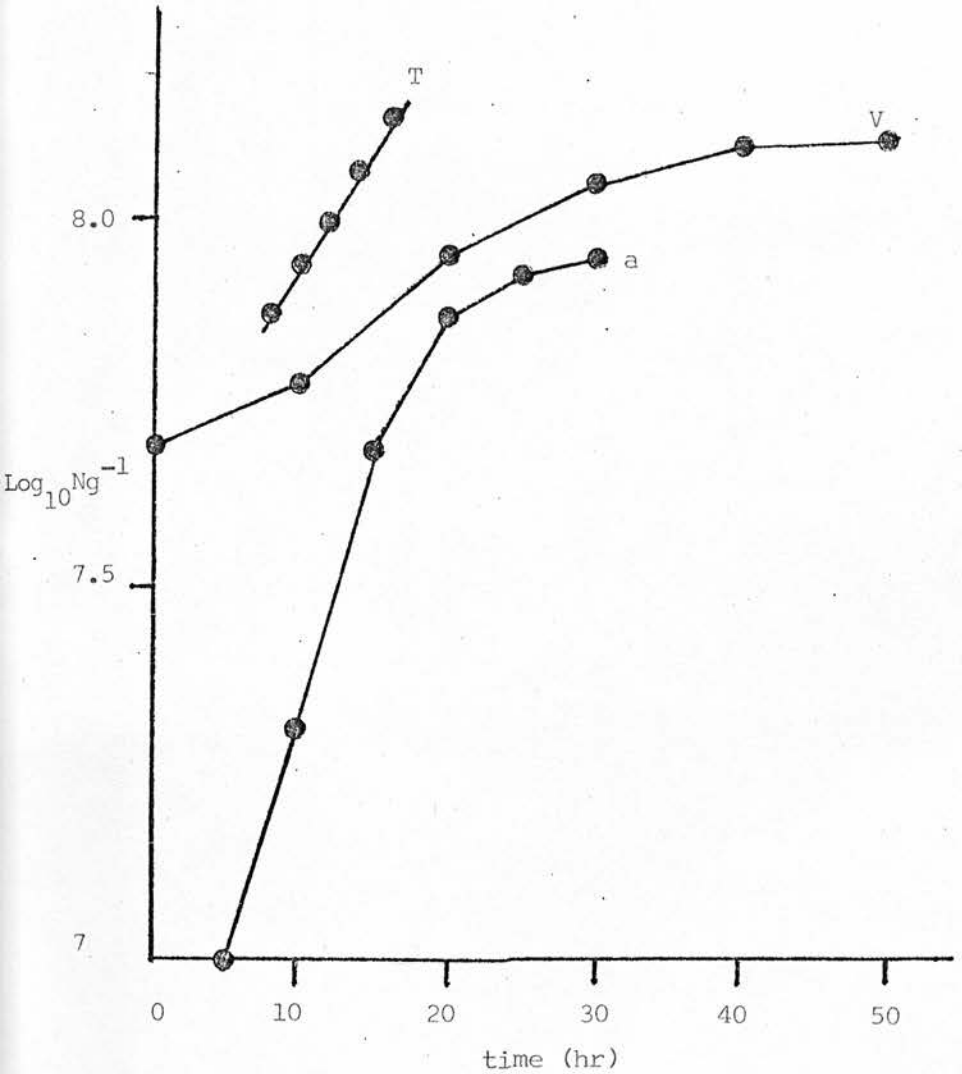


Figure 10. Logarithmic plot of T, V and a after a growth promoting stimulus at time 0.

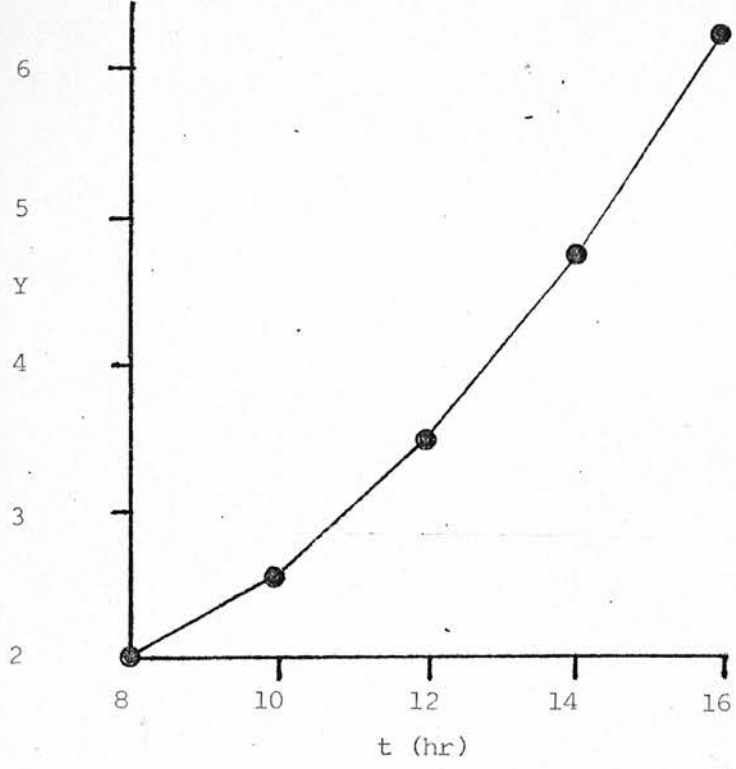


Figure 11. Calculated derivation of μa_t (Equation 16); see text, p. 65.

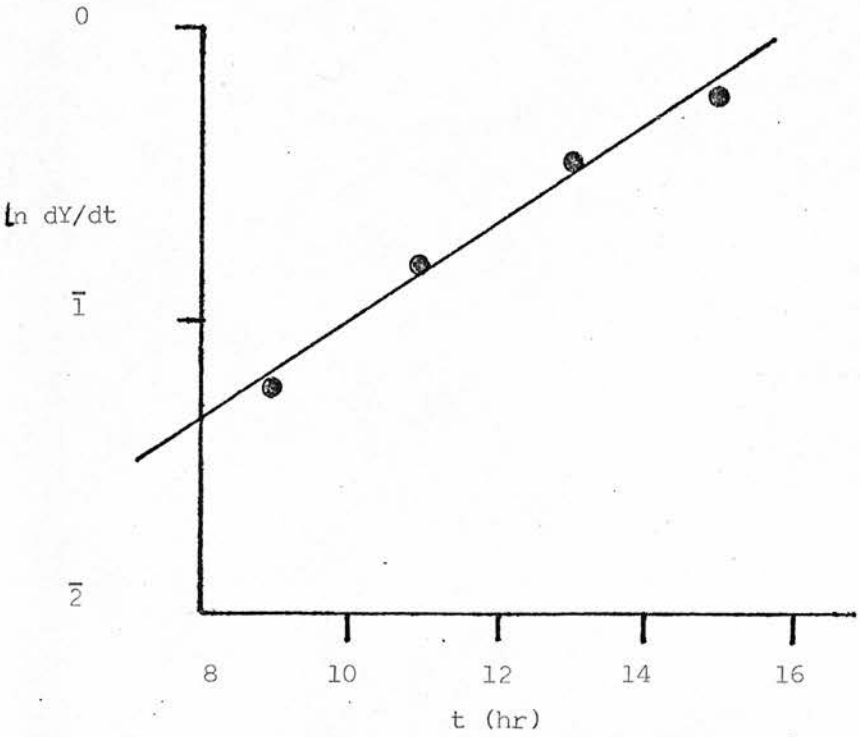


Figure 12. Calculated derivation of ln μa_o (Equation 16); see text, p. 65.

of populations V and a was envisaged. These populations were modelled by differently genetically labelled strains of the same species of bacterium. Labelling took the form of antibiotic resistance so that the two populations could be cultured separately on selective media from a mixed inoculum. It was intended that experiments with homogeneous liquid cultures should be followed by studies of soil inoculated with various proportions of a and V. In fact the physical model proved to be non-functional because of the genetical nature of the organism chosen. Consequently, the applicability of the mathematical model could not be tested.

A (2) The choice of experimental organism

The choice of organism was governed by facility of manipulation and reproducibility of behaviour. The organism must:

(a) be easy to grow in a defined medium;

(b) be rapidly identifiable;

(c) be simply mutable;

(d) not possess spores or cysts;

(e) not exhibit pronounced multicellularity, filamentous growth or clumping.

Many bacteria conform to these requirements; the one chosen was Pseudomonas oxalaticus (Khambata and Bhat, 1953). This organism uses formate as a carbon and energy source by means of a modified Calvin cycle (Quayle and Keech, 1959). Carbon is assimilated as CO_2 which is produced as a result of respiration at the expense of formate. The defined formate medium (Quayle and Keech, 1959) on which the organism grows provides a selective environment in which few chance contaminants can grow. The two formate utilising strains of Ps. oxalaticus (OX1 and OX23) isolated by Khambata and Bhat (1953) may be differentiated by their utilisation of glucose and ethanol; both may be recognised on a diagnostic oxalate containing medium (Bhat and Barker, 1948). These factors coupled with the the cytological and physiological attributes of the pseudomonads were judged to warrant the use of this species as the experimental organism. Cultures were obtained from the National Collection of Industrial Bacteria (OX1 = NCIB8642; OX23 = NCIB8643).

A (3) Isolation of antibiotic-resistant mutants of Pseudomonas oxalaticus OX1

For efficient separation of the components of a two-organism population, each component must be resistant to antibiotics to which the other is sensitive. Ideally, the mutants used should have arisen spontaneously so that the genotypes of the organisms would be as similar as possible. This ideal could not be realised and it was necessary to induce some of the mutations with nitrosoguanidine (NTG).

Materials and Methods

Antibiotic sensitivity was determined on 0.1% yeast extract nutrient agar (YENA) plates containing the antibiotic over a range of concentrations. Cultures were grown in nutrient broth (Oxoid) supplemented with 0.1% yeast extract (YENB).

Dilutions of exponential phase YENB grown cells were made in antibiotic-containing YENB. These cultures were incubated several days to allow enrichment of resistant mutants. The presence of these was inferred from increasing turbidity in the culture vessels and was confirmed when subcultures grew on YENA containing the antibiotics. Inocula from the enrichments were spread onto antibiotic containing gradient plates and those colonies selected which were resistant to the greatest antibiotic concentration. Enrichments were set up in quintuplicate at twice and four times the minimum inhibitory concentrations (MIC) of antibiotic previously noted for the wild type.

For induced mutants, washed suspensions of OX1 were buffered to pH5 (citrate-phosphate, 0.2M) and pH8 (tris-HCl, 0.2M). NTG was added to these suspensions to a terminal 100 µg/ml. The suspensions were then vigorously shaken at 18°C. 0.1 ml samples were removed after 10, 20 and 40 minutes and used as inocula for 10 100 ml aliquots of YENB. After 16 hr incubation to allow expression of the mutations, triplicate antibiotic gradient plates were set up as above.

Results

Multodisc sensitivity tests showed that the wild type was sensitive to the following antibiotics: erythromycin, novobiocin, ampicillin, bacitracin, benzyl penicillin, polymyxin, neomycin, chloramphenicol, streptomycin and chlortetracycline. When these antibiotics were tested over a range of concentrations, certain of them although inhibitory allowed substantial growth at high concentrations (250 µg/ml). Such antibiotics were not considered further (erythromycin, novobiocin, bacitracin and benzyl penicillin).

Attempts were made to obtain spontaneously arising mutants resistant to the remaining six antibiotics. The experiments succeeded in obtaining mutants resistant to each single antibiotic. The mutagen NTG was then used to

increase mutation frequency. This procedure resulted in the isolation of mutants resistant to two antibiotics. Mutant cultures which could grow in defined formate medium and maintain uniform antibiotic resistance in the absence of the drug were selected. This necessitated rejection of all polymyxin resistant 'mutants'. MICs for the remaining antibiotics with respect to the wild type OX1 were as follows:

neomycin	2.5 μg/ml (in YENA)	N*
chloramphenicol	20 "	C
ampicillin	10 "	A
chlortetracycline	10 "	TC
streptomycin	5 "	S

(* code letters by which these antibiotics are subsequently referred to).

After testing for stability of resistance, a number of potentially useful mutants were obtained:

Index Number	Working concentrations*
CS169	C50 S10 μg/ml in YENA
CN388	C50 N9 "
NS135	N4.5 S5 "
AN393	A100 N18 "
AS404	A25 S5 "
AC397	A50 C50 "
TCC420	TC25 C25 "
TCN416	TC100 N18 "
TCN418	TC50 N9 "

(* antibiotic concentrations allowing mutant but not wild type growth).

A (4) Examination of antibiotic-resistant mutants of *Pseudomonas oxalaticus* OX1

Before using mutants in mixed cultures it was necessary that they could be independently assayed from mixtures. A series of separation tests was set up as described below. At this point the experiments became abortive in that mutants could not be satisfactorily separated from mixtures.

Materials and methods

For quantitative separation the two mutants were grown in defined formate medium and the culture density estimated spectrophotometrically. Mixtures of the two cultures were made to contain a variety of proportions of each member. After mixing, inocula were plated on antibiotic-containing YENA homologous to each component in the mixture. YENA cultures gave a sum count of the two components; density of the parent culture was also determined so that the proportion of each component in the mixtures could be calculated from the volumes used.

Results

(1) Separation test on TCC420/NS135 and TCC420/AS404

Mixtures of 100/0, 95/5, 75/25, 50/50, 25/75, 5/95 and 0/100% of each component were made. After 3 days incubation at 28°C no growth was visible on any of the chlortetracycline-containing plates. It was later found that none of the TCC-resistant organisms had retained their resistance to the drug on subculture in its absence.

This was at variance with earlier results of resistance-retention experiments and no explanation was forthcoming.

(2) Separation test on NS135/AC397

The plate counts* obtained from the experiments are shown in Table 1:

Mix No.	AC397	NS135	YENA	AC397%exp.	NS135%exp.
1	0	1.16**	1.03	0	100
2	2.4	2.4	-	50	50
3	3.4	2.5	2.45	57.5	42.5
4	3.7	-	5.95	62	38
5	6.5	0.61	6.07	91.5	8.5
6	11.5	0.07	8.34	99.41	0.59
7	15.7	0	13.6	100	0

(*All counts are means and x 10^{-6}/ml mixture; ** mean count not significant - count on two plates out of three less than thirty.)

Ideally the sum (AC mean + NS mean) should be equal to the mean YENA count for each mixture. The ratio of the difference of the two means to the variance of the difference should not exceed 1.96 or 1.645 if the difference is to be considered insignificant at the 5 or 10% levels respectively (Bailey, 1959).

Hence,

$$d = \frac{/\bar{x}_3 - \bar{x}_4/}{\bar{x}_1/n_1 + \bar{x}_2/n_2 + \bar{x}_3/n_3} \qquad (17)$$

where,

$\bar{x}_3$ = mean YENA count

$\bar{x}_1$ = mean AC count

$\bar{x}_2$ = mean NS count

$\bar{x}_4 = \bar{x}_1 + \bar{x}_2$

$n_1 \ldots\ldots$ = number of replicate plates

All differences except that of mixture 3 were insignificant at the 10% level. Since control mixtures 1 and 7 showed that no growth of NS135 occurred on ACYENA and no growth of AC397 occurred on NSYENA the possibility of a lack of discrimination between components of the mixture was unlikely. However, since (AC +NS) counts were consistently higher than the control YENA counts the media did not appear to discriminate as intended.

The above experiment was repeated with greater attention being devoted to mixture and parent culture homogenisation. All known potential sources of manipulative error were controlled as carefully as possible. When the plates from the repeated separation test were examined, growth was so sparse on the antibiotic-containing plates as to render analysis impossible. It was found that as in the previous case, reversion to sensitivity was responsible. NS135 had lost its resistance to neomycin on subculture in its absence. Under the same circumstances, AC397 had reverted to wild type sensitivity to both ampicillin and chloramphemicol. Consequently, these two mutants were discarded.

Separation tests on AS404/CN388 and AN393/TCC420 were also discontinued when the organisms failed to behave predictably.

(3) Re-examination of the antibiotic sensitivities of OX1 mutants

The anomalous results described above necessitated a re-evaluation of the stock of mutant organisms. All mutants were transferred successively from YENB to YENA five times in the absence of antibiotics. They were then tested against all the antibiotics used; the results are summarised below:

Table 2. Re-examination of antibiotic-resistant mutants

mutant No.	supposed resistance	resistance found
393	A,N	N,C,A,S
169	C,S	C
388	C,N	none
135	N,S	S
404	A,S	S
397	A,C	none
420	TC,C	C,A,N
416	TC,N	none
418	TC,N	N,C,A,S

388, 397 and 416 had reverted to wild type sensitivity; all mutants originally resistant to chlortetracycline (TC) had lost this resistance. 393, 418 and to a lesser extent 420 presented a puzzling appearance in that they were resistant to antibiotics to which they had never been previously exposed.

A (5) Discussion

The mutant system described is completely unsuitable for the purpose intended. This was the result of an unfortunate choice of experimental organism. Retrospectively it would have been preferable to have chosen an organism whose genetics had been well studied. It would be advisable in future experiments of this type to use E.coli K12 and to take advantage of strains which have already been altered in a known manner. It is concluded that the idea of using a mutant system to study the applicability of the (V + a) estimation method to soil systems is still feasible.

An alternative approach would be to use organisms of two species which could be separated on specific media (possibly antibiotic-based). In this case the basis of interaction between the two organisms would have to be carefully explored. Such an approach would lose one important advantage of the mutant system. This is that the latter system consists of a model community made up of two populations which differ only in the nature of a very small number of genes. In such an experimental system, the ecological effects of genetic variation could be studied in a controlled manner in the knowledge that the biochemical basis of interaction may be predicted from the population level to the level of gene function. To study such interactions as predation, clearly another type of organism would have to be introduced to the system. In

this way a series of community models of increasing complexity could be built up.

The failure of the mutant model system has not detracted from the usefulness of the mathematical method it was set up to investigate. In a real soil system, the choice of a suitable indicator population to provide the V component data would be of great importance. The conditions under which the indicator could be enumerated would have to be highly defined and made as specific as possible. However, speculation on the usefulness of the V + a estimation method or the mutant model system approach is unwarranted in the absence of data.

SECTION B

Examination of some techniques for studying soil microbial community dynamics

Any study of soil microbial community dynamics may be resolved into three basic problems:

(1) What aspect of community dynamics should be studied?

(2) What sort of soil samples should be studied?

(3) What techniques should be used?

These problems are interdependent and the first two are discussed at length later (p.11,128). Whatever answers are chosen for them however, problem (3) becomes an assessment of population or community growth; this is estimated by the measurement of such growth-correlated parameters as cell numbers, biomass, activity etc. The use of population or community activity in studies of dynamics has not been well exploited and is discussed later (p.164). There is no direct method for measuring microbial biomass in soil; this is an inevitable result of the size of the organisms, their numbers and variety and of the fact that procedures for extracting and purifying them from soil are at present primitive.

Chemical methods of microbial biomass measurement are not sufficiently advanced to be used on a routine basis. Attempts by Millar and Casida (1970a) to estimate biomass from soil muramic acid content have given rise to estimates of 5.6 μg muramic acid/10^6 plateable cells. Pure culture studies show muramic acid contents of 0.004 $\mu g/10^6$ gram positive bacteria, 0.0005 $\mu g/10^6$ gram negative bacteria and 0.066$\mu g/10^6$ aerobic bacterial spores. This variation in muramic acid content between different types of bacteria makes it impossible to know whether

high soil muramic acid content indicates high biomass concentration, use of muramic acid as a storage material or extraction of muramic acid from dead as well as living cells. (The last possibility appears most likely.)

Adenosine triphosphate concentration is used routinely in estimation of microbial biomass in aquatic systems (Sorokin and Kadota, 1972). In soil, the contributions of ATP from micro-arthropods, nematodes, crustaceans, protozoa, algae and other fauna would prevent interpretation of soil ATP content as microbial ATP content.

The soil sterilisation/reinoculation method of Jenkinson (1966) depends on the use of killed soil micro-organisms as nutrients by an added inoculum. The flush of CO_2 produced on inoculation is a function of the killed biomass. Each determination however, requires a period of 10-20 days incubation and large soil samples (ca. 300g). The method is thus not practicable for small-scale laboratory experiments where each soil sample may be only 5-10g.

The only techniques currently available to soil microbiologists are those concerned with enumeration. Suitable estimates of numerical population density may be converted to biomass terms if a satisfactory mean cell weight or biomass frequency distribution can be obtained. There are extant, only two enumeration methods - the microscopic count and the cultural count. There are perhaps as many variations on these methods as there are soil microbiologists which is an indication of the degree of imperfection of the methods. The object of the present section of this study was to examine briefly the more popular methods of enumeration.

B (1) Microscopic counting methods

(a) Theory and historical background

All methods of this type attempt to derive from soil a representative sample containing all the types of micro-organisms present. A series of subsamples from this main sample are examined microscopically and from a knowledge of the weight of soil contained in the subsamples, an estimate of the cell density in the original soil is made. The earliest use of such a method is generally attributed to Conn (1918); later Winogradsky (1925), Thornton and Gray (1934) and Jones and Mollison (1948) made significant advances in the methodology. Alongside these strictly quantitative methods a variety of microscopic procedures were evolved to gain some qualitative insight into the arrangement and form of soil micro-organisms. Such methods were not considered in this study.

A wide variety of experimental procedures has evolved from the work of the early authors. Methods using the electron microscope are at present in their infancy but show some promise (Nikitin and Makarieva, 1970; Nikitin, 1973) in that examination of soil at elevated magnifications has revealed a large range of new micro-organisms difficult or impossible to study by optical microscopy. (Nikitin, 1964, 1973; Mishustin and Nikitina, 1973). Although the pedoscopes of Perfiliev and Gabe (1969) were not primarily intended for quantitative studies, it appears that they may be applicable (Nikitin 1973); reference of count data from this source to physical soil parameters could present serious difficulties since a micro-habitat of specific size is studied in the capillary.

Most microscopic counting methods however use optical (or u/v fluorescence) microscopy and are carried out on smears of soil suspension or on films prepared from suspension and agar solution mixtures. The use of smears is necessary when the use of fluorescent dyes is adopted since agar exhibits a strong auto-fluorescence (Casida, 1967; Babuik and Paul, 1970). Uneven drying of soil smears can cause problems in the interpretation of counts; thus after drying, the distribution of micro-organisms on the slide becomes non-random. This was recognised by Thornton and Gray (1934) who used a suspension of indigotin particles added in a known ratio (w/w) to the soil. Comparison of the ratio of micro-organisms to indigotin particles and the known indigotin/soil ratio allowed distribution difficulties to be circumvented. This method has not however been extensively adopted by subsequent workers. (An exception was the study of Taylor (1936) already referred to). In studies on soil smears with non-fluorescent dyes an agar overlay is usually placed on the smear; this layer prevents the disintegration of the film during staining and washing manipulations. It however introduces a problem shared by the agar film technique in that a high and often variable background cell count is obtained from the agar. This has apparently received publicity only recently (Harris, 1969; Rovira pers. com. 1972). Harris (loc.cit.) has provided a simple means of calculating the "uncontaminated" soil count; this however necessitates calibration of the agar which represents considerable extra work. A more satisfactory alternative procedure was evolved in this study and is described later.

Although many modern studies using microscopic counts utilise soil smear preparations, the method described by Jones and Mollison (1948) possesses many advantages. Its main disadvantage is the meticulous care and patience required in its execution. The procedure consists of the dilution of a soil suspension in an agar solution. The mixture is moulded as a block of known thickness in a haemocytometer. After setting, it is removed and mounted, allowed to dry to a film of negligible thickness whereupon it is stained and examined. The distribution problems referred to above are thus avoided and the precise weight of soil examined in a specified microscope field simply calculated. Other technical advantages of the method are discussed later (p.109).

In the absence of a perfect method of enumeration, assessment of the accuracy of methods of estimating cell numbers in soil is difficult or impossible. Jones and Mollison showed however that on addition of a known number of bacteria to a sterilised soil of known bacterial content, recoveries of 96-98% of the added organisms could be obtained by their method. The real situation is naturally more complex and 'losses' of cells as a result of failure to remove them from opaque soil particles represents an unknown loss of accuracy. The same reasoning applies to organisms associated with larger mineral particles which are not included in the final suspension. The precision of the method is easily investigated experimentally and the optimal experimental procedure and number of replicate samples, preparations and fields examined may be readily determined. Thus

the maximum precision commensurate with effort expendable may be calculated. Quenouille (Jones and Mollison, 1948) found that the distribution of single organisms in fields of view took the form of a negative binomial expansion; however, Prasad (1968) using a similar system found a Neyman distribution while according to Trolldenier (1973) the distribution of organisms in soil smears was Poissonian. Although further research is warranted in this area, as a first approximation standard errors and confidence limits are normally calculated according to a Poisson distribution. More often however microbial counts (total and viable) appear in the literature without estimates of their precision. Each stage in the preparation of soil for microscopic examination is open to a number of sources of error; these were considered and steps taken to arrive at a standardised procedure.

(b) Soil homogenisation problems

These are in part similar to the problems experienced in preparing suitable suspensions of micro-organisms for dilution prior to colony counting procedures (p.100). In some requirements however, direct counting methods are not so stringent as plate count procedures. For example, the requirement that each colony counted arose from a single organism necessitates complete disintegration of all aggregates of propagules. (In practice, this may never be achieved - see p. 101). In microscopic counting procedures, only potential propagules need be considered and provided the number of organisms within them can be counted, this does not represent a significant source of error. It does however alter the expected frequency distribution of the data

(according to Jones and Mollison from a Poissonian to a negative binomial) and hence the calculation of the inherent limits of precision of the method.

The basic requirements of a homogenisation procedure are that the maximum possible number of micro-organisms should be removed from the soil particles and suspended in a suitable diluent (Eliade et al, 1966). The number of micro-organisms in suspension should be such that they can be readily counted while the background of inanimate soil material should be as low as possible. A number of mechanical methods of soil disintegration were investigated; these are described below. In this study distilled water, P.B.S. (0·2M phosphate-buffered saline, pH 7·6) and calgon (0.2% aqueous sodium hexametaphosphate) were used as diluents during soil disintegration. The role of the latter in desorbing micro-organisms from clay minerals was described by Marshall (1971).

The effect of mechanical shaking on a soil suspension was investigated by the use of a 'Griffin' wrist-action flask shaker. It had been established that 10 min shaking at half maximum speed of a suspension consisting of 1 g (moist weight) seived (3 mm) garden soil in 9 ml calgon gave higher total and plate counts than a variety of other treatments. Consequently this was taken as the starting point in the preparation of material for microscopic examination. Four smears (p. 87) were prepared from 0.02 ml of the homogenate; staining was carried out for one hour in phenolic aniline blue (p. 91). A graticule delimiting an area of one square centimetre was inserted in the microscope eyepiece and the number of bacteria per field was recorded until a

significant number of organisms (>300) had been noted on each slide. No attempt was made to convert this data to counts on a soil weight basis. For the purpose of comparison between preparation methods on the same soil sampled at one time, the mean count per field was adequate provided the field dimensions remained unaltered. In this case, a mean of 42.9 organisms per field was found. The background of soil particles was such that occasional fields had to be discarded because of masking by inanimate material. This was not sufficiently serious to prevent an excess of suitable fields being found.

The potential of ultrasonic vibration as a method of soil dispersion (Whittles, 1923; Stevenson, 1958; Zvyagintsev, 1968; Clarke and Hill 1970) has recently been reviewed by Watson (1971). The technique was investigated as follows: An M.S.E. ultrasonic probe vibrating at 18kHz provided the energy source in these experiments. During sonication, a soil/calgon mixture (prepared as above) was maintained close to 0°C in a stainless steel beaker surrounded by an ice bath. (It was found that plastic vessels retained sufficient heat on sonication to allow the temperature of the suspension to rise to about 60°C in four minutes.) Four replicate smears were prepared after 2, 4, 8 and 12 minutes treatment. The results are shown in Figure 13. The procedure is clearly less efficient than mechanical shaking. It is clear from Figure 13 that physical destruction of cells has taken place as exposure time increased. The levelling off in the graph is probably indicative of the presence of organisms which were either physically protected from the vibration or were resistant to disintegration. Such organisms could be spores, cysts or other cells with strengthened cell walls. Since it was clear that cells as well as soil structure were destroyed by sonication, the technique was not pursued.

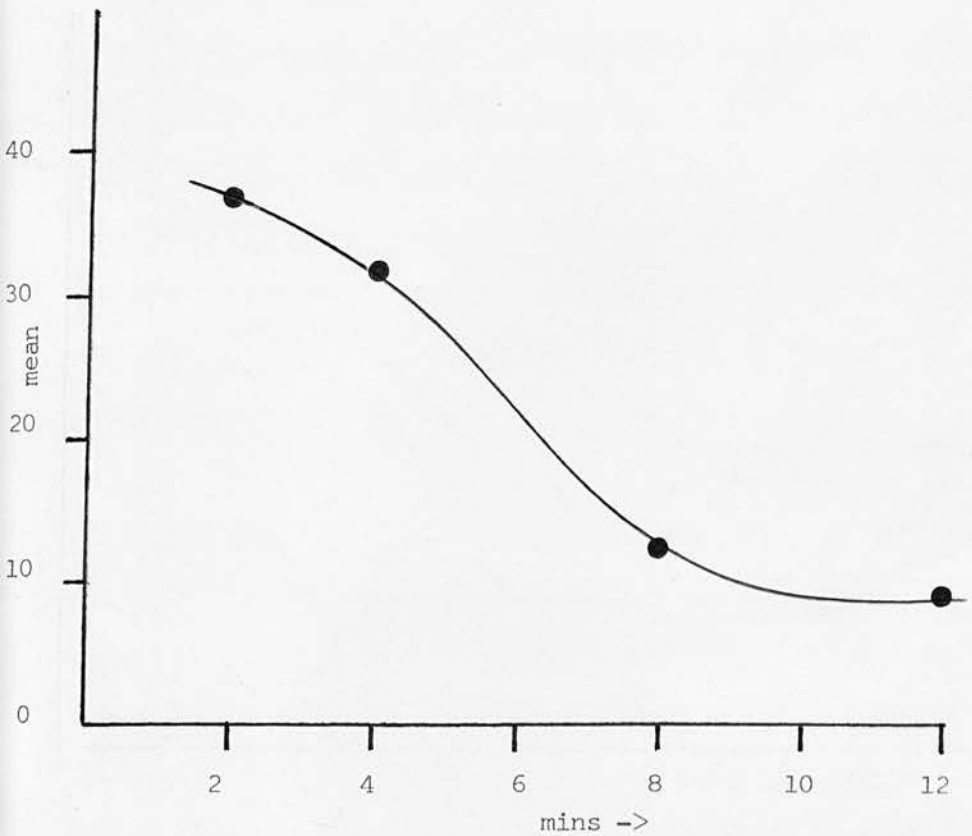


Figure 13. Variation in mean count/field with time at 18 k Hz.

Another possible means of aggregate disintegration is the use of a mechanical blendor. An M.S.E. miniature tissue homogeniser was compared to the standard shaking method. Blending of the soil/calgon mixture was carried out for 1, 2, 3 and 4 minutes. In no case was the count greater than half that obtained by shaking. The aggregate disintegration procedure described by Jones and Mollison (1948) involved grinding the samples in a mortar and pestel. This was tried but it was found to be very difficult to reproduce identical treatments on successive samples. As a result of the series of experiments, a ten minute period of mechanical shaking was adopted as the routine aggregate disintegration procedure. Extended shaking times were not found to increase the count significantly.

The possibility exists of reaggregation of cells after disintegration. There is little that can be done at present to prevent this. However, experiments on the numbers of cells in clumps observed in stained soil preparations (p. 101) showed a relatively constant number of cells per clump. This indicates that if reaggregation occurs, it is not likely to interfere with the accuracy of total counts provided the distribution of cells within clumps is considered. Jones and Mollison (1948) found that the frequency distribution of clumps of cells was Poisson; that of the number of bacteria per clump formed a logarithmic series, hence the total number of bacteria per field fell into a negative binomial distribution.

(c) Preparation of smears and films.

0.01 - 0.05ml of soil suspension were spread over 2 cm^2 on chloroform-defatted microscope slides. Sometimes the area on the slide was outlined by a wax pencil. The suspension was allowed to dry at room temperature.

In order that dried smears might retain their integrity it was necessary to place a thin film of agar on top of the suspension. This was allowed to dry completely before staining.

Smears prepared as described above were found to be very useful for rapid qualitative examinations of soil. Their value in quantitative work was however questionable. This was largely the result of the way the original soil suspension droplet dried on the slide. It is impossible to dispense a soil suspension from a pipette uniformly over a flat area; this meant that mixing of the drop on the slide was necessary - a task difficult to carry out reproducibly. Because of the convexity of the meniscus of a water droplet on a non-wettable surface, the film of suspension was always much thinner at the edges than at the centre. Consequently, drying proceded from the edges inwards; small particles of bacterial dimensions tended to accumulate at the edges (probably as a result of convection currents towards the area of greatest evaporation coupled with an entrapping surface tension effect in the thinner parts of the film). Larger particles of soil mineral or organic material tended to accumulate at the centre. This process resulted in a non-random arrangement of bacteria over the delimited area rendering adequate sampling of representative microscopic fields difficult. When smears were used the problem was approached by taking transects across the entire smear area. (This is in opposition to the procedure adopted by Trolldenier (1973) who advocated sampling only at the edges of the smear).

Distributional difficulties are largely avoided in the Jones and

Mollison agar film technique; consequently attention was directed towards this method. Disaggregation and dilution were carried out much as described for the soil smear technique. Ca. 2.5 g (wet weight) of soil was homogenised in 5 ml diluent. After allowing the larger particles to sediment for 3-5 sec, the supernatant was decanted. The sediment was washed clean with successive 5 ml aliquots of diluent and the combined supernatants made up to 50 ml with agar solution (p 94) at 60°C. From a knowledge of the number of 5 ml aliquots of diluent used the exact weight of agar added was calculable. This was necessary when account was being taken of the background count originating from the agar. In later work where agar with no background was used, 1 ml of the combined supernatants was mixed with 2.5 ml 2% w/v agar solution. (This agar concentration was critical for the mechanical properties of the film.). The mixture was subjected to vigorous mechanical shaking for one minute prior to moulding. Because of the viscosity of the suspension, sedimentation of the larger components was retarded. When the suspension was pipetted, no problems were experienced with accumulated sediment in the lower tip of the pipette; in purely aqueous suspensions (as used for the smear method) this was found to be a major problem.

Samples of the suspension were pipetted into the depression of a 0.1 mm depth (Gallenkamp) haemocytometer. The depression was closed by means of a suitably inflexible optically plane coverslip. The pressure exerted on the coverslip by a 50 g weight was used uniformly. For uniform film thickness and cleanliness, it was necessary to wash the haemocytometers with mildly abrasive

detergent powder between each use and to preheat them to 45°C before the addition of the suspension. The volume of suspension was carefully controlled so that excess did not overflow the drainage channels at the side of the depression, (i.e. there was glass to glass contact between slide and coverslip).

Five replicate preparations were made from each suspension and held at 4°C for 5-10 minutes to effect complete film solidification. Coverslips were removed by lateral pressure so that there was little tendency to tear or dislodge the film. A rectangular section was cut in the film by vertical incisions of a razor blade, a small portion of the film being left attached to the waste material in the drainage channels. This precaution was necessary to prevent material of uncontrolled thickness from contaminating the film. Waste material was removed and the haemocytometer submerged in clean distilled water. The film was dislodged by gentle manipulation with a broad soft camel-hair brush and floated onto a standard (Chance No.2, 25 mm^2) coverslip. Air bubbles in and on the preparation were avoided by carrying out all these manipulations below the water surface. The mounted film was placed on a piece of filter paper and all free water soaked away. It was found that if this was not done, excessive drying times were sometimes necessary allowing microbial proliferation in the film.

At this stage the films were fixed where necessary (below) and allowed to dry at room temperature (optimally 1/2-1 hr). When dry they were of negligible thickness (within the depth of focus of the 100X microscope objective used). Staining was carried out

and the washed films again allowed to dry. The coverslips were lowered film-side down onto a drop of 'Euparal' mountant on a clean slide. It was found necessary to mount the unstained films on coverslips as described because the thickness of the film plus the mountant and a coverslip sometimes exceeded the focal length of the objective, when the films were mounted directly onto the slides.

(d) Staining and fixing of films and smears

Fixation of soil smears serves primarily to cause the micro-organisms to adhere to the slide. This was most simply effected by the normal heat treatment; alternatively an agar overlay was used as described earlier. Fixation of Jones and Mollison preparations was carried out by exposure of the mounted wet films to the vapour from a few drops of 40% w/v formalin placed in the lid of a closed petri-dish. The staining method prescribed by Jones and Mollison (1948) was found to be very satisfactory. The stain, phenolic aniline blue (P.A.B.) was prepared from glacial acetic acid (4 ml), 1% w/v aniline blue (1 ml), and 5% w/v aqueous phenol (15 ml). A staining period of 1 hour was found to be satisfactory with no increase in contrast when exposure was increased up to 6 hours. The stain was found to be equally useful for smear and film preparations. The stain tended to precipitate on exposure to air; consequently the nature of the staining vessel assumed some importance in terms of conservation of solution. The cubical lids from boxes of Chance No.2 25 mm^2 coverslips proved satisfactory in this respect; a 0.5 cm layer of paraffin wax was placed in the base of such a box and coverslips bearing films inserted edge-on. When solid the wax held the films

firmly; they could then be stained in a minimum volume of stain. The method also allowed replicate preparations from the same sample to be kept together and receive identical treatment. Smear preparations on ordinary slides were stained in large petri-dishes.

An alternative staining method was also tried. Carbolic erythrosin has been advocated by a number of Russian soil and aquatic microbiologists (e.g. Sorokin and Kadota, 1972). With this method, staining intensity was found to increase up to 6 hours treatment. Even after this period however, contrast was inferior to that obtained by the PAB method. It was also found that many bacteria to yeast-sized spots of stain were left on the slide. A similar but particulate deposit had been noted with PAB and was best removed by membrane filtration of the stain prior to use. It was found that washing stained preparations in water served no useful purpose and excess stain was removed by rapid rinsing of the preparations in absolute alcohol. This process also assisted in dehydration and was adopted routinely.

(e) Treatment of agar for the preparation of films

The problem of background counts in agar has already been mentioned. Harris (1969) found between 10^7 and 4×10^9 organisms/g of various agar powders. In terms of agar added to a preparation this would often lead to an insignificantly small error in count; however, in cases where a low count from the soil and a high count from the agar coincided errors could become very much significant. Initially control films containing no soil were set up to make allowance for this. This was technically difficult;

films containing no soil are virtually invisible in water and large numbers were inevitably lost in attempts to mount them. This difficulty was overcome by adding sufficient (membrane-filtered) fuchsin solution to colour the agar. Such procedures however involved extra microscopic examinations and were time consuming. Consequently a means of removing the bacteria from agar was sought.

It was found possible to filter molten 2% agar solution through a 0.3 μm pore size Millipore membrane under reduced pressure. The complete filtration flask, funnel and filter were placed in a 105°C oven for 20 minutes prior to use; on removal the apparatus was lagged heavily with cotton wadding and used immediately. The agar solution was heated to 120°C in an autoclave, the pressure rapidly released and the solution filtered while as hot as possible under reduced pressure. In this way it was possible to filter 25-50 ml batches of agar although cooling of the apparatus was a major problem. The filter flask and all containers subsequently coming into contact with the agar were rinsed in chromic acid to destroy adsorbed micro-organisms. The agar was aseptically dispensed hot into sterile vessels. Agar prepared in this way and stained as described contained no detectable microbial background.

Filtration as described was tedious and unsatisfactory and an alternative method of maintaining the temperature of the apparatus was sought. It was found that by use of a heated water bath, the temperature of the flask could be maintained close to 100°C while that of the funnel containing the agar

remained between 90 and 100°C. Even under these conditions filtered agar solidified almost immediately it passed through the membrane. This was thought to be partly due to an adiabatic expansion effect (with consequent local cooling) and partly due to evaporation of water under reduced pressure leading to local cooling as a result of loss of latent heat. Consequently a means was sought of supplying heat directly to the filter membrane.

Electrical heating elements were made from soft iron wire insulated in fibreglass. One element was wrapped tightly round the base of the funnel and the other wrapped round the filter carrier itself prior to assembly. The two heaters were wired in parallel and their resistance adjusted (via their length) so that they drew 25 watts when connected to a transformed mains power supply (6 v). It was found that when this method was used only electrical heating was necessary. The funnel was lagged with cotton wadding and it was found that 150-200 ml of agar could be filtered before it was necessary to replace the membrane. Subsequently all agar used in the preparation of Jones and Mollison films was filtered in this way.

(f) Discussion: The use of total count procedures

It was concluded for the reasons mentioned above that the agar film method as modified was superior to the soil smear method for quantitative work. Consequently no attempt was made to study fluorescent dyes which require the use of smears. No statistical evaluation of the method was judged to be necessary in the light of the treatment by Quenouille (Jones and Mollison, 1948). In use, the field of view and terminal suspension dilution were

adjusted so that an average of 10-20 organisms per field were visible. Ca. 300 organisms per film were counted and routinely four films per suspension sample examined. It may be noted that many of the criticisms levelled against the method (Parkinson, 1970; Parkinson, Gray and Williams, 1971) to the effect that no qualitative information on microbe/microhabitat relationships is gained is inappropriate. The method is not designed to gain such information; nor is it designed to differentiate between active and inactive or dead organisms. It attempts to estimate the total number of micro-organisms (irrespective of condition) in a soil sample. Within the limits of optical microscopy it is eminently successful in this aim.

Attempts have been made to differentiate living and non-living cells on the basis of acridine-orange uptake (Strugger, 1948) and subsequent fluorescence. It appears now that early conclusions based on this method are spurious and in reality it can only be applied to some gram negative bacteria under conditions of uniform dye concentration (Bucherer, 1966). Attempts to achieve a similar end by the use of a colorimetric enzyme assay are described later (p.111). Many workers appear to be discouraged from using the Jones and Mollison technique because of the difficulties encountered in uniform film preparation and interpretation. It is of interest therefore to illustrate some of the range of organisms which may be visualised by the method and to present examples which may be considered the result of good technique.

Such examples are presented in Plates 1 - 6. Examination of Plate 1

recalls criticisms often made of the technique that it is impossible to identify micro-organisms visually. It should be emphasised that this is not an intention of the method nor has it been desirable to identify organisms in this study. Thus the ellipsoid cells illustrated may be yeasts or fungal spores; it is possible that they are bacteria. The associated bacillary elements are almost certainly bacteria. The crystalline nature of much of the soil mineral material is also apparent in this photograph. In Plate 2 it is interesting to note the colonisation of the (probably dead) diatom by a number of small coccal bacteria. Above and to the right of the alga is a stained earthworm seta; this is distinguishable from fungal mycelium by its characteristic curve and tapering apex. Diatoms can be found in most soil horizons; in the garden soil examined in the preparation of Plate 2, this distribution is probably a result of tillage. In Plate 3, a testate amoeba (alive at the time of specimen preparation) may be clearly seen. The test and cell nucleus have stained clearly. Few protozoa other than testates and a variety of encysted forms survive the homogenisation procedure necessary for sample preparation. Flagellates, ciliates and naked amoeba are however demonstrable culturally (p. 104).

Cocci like those in Plate 4 are very commonly observed in most soils. Their adherence in clusters is of interest since steps have been taken to disintegrate such aggregates. This could suggest the presence of some unstained extracellular binding material originating from capsular or other cell-produced substances. (See also Plate 6). Amorphous black humic material

representing soil organic matter is obvious in Plates 4, 5 and 6. In Plate 5 an actinomycete can be seen growing out of such a substrate. In this case the organism could be very tentatively ascribed to the genus Micromonospora. Plate 6 is also an example of the exception to the rule; here part of a vegetative swarm of myxobacteria is clearly recognisable. This type of micro-organism may be recognised by the alignment of the cells within the group, their association in groups (probably correlated with known production of extracellular mucilage) and their relatively poor staining compared to eubacteria.

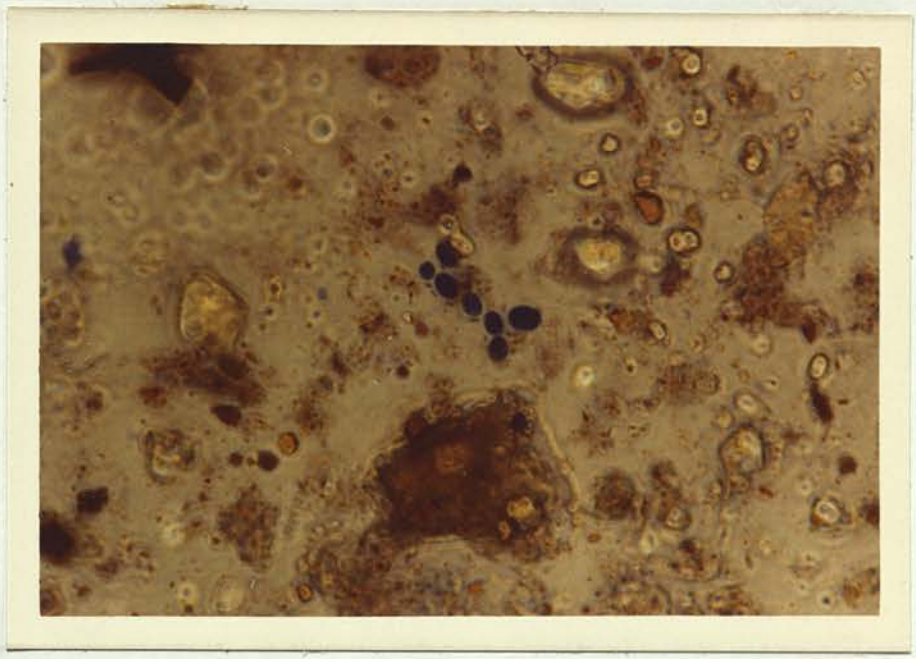


PLATE 1 ⊢ ≡ 10 µm

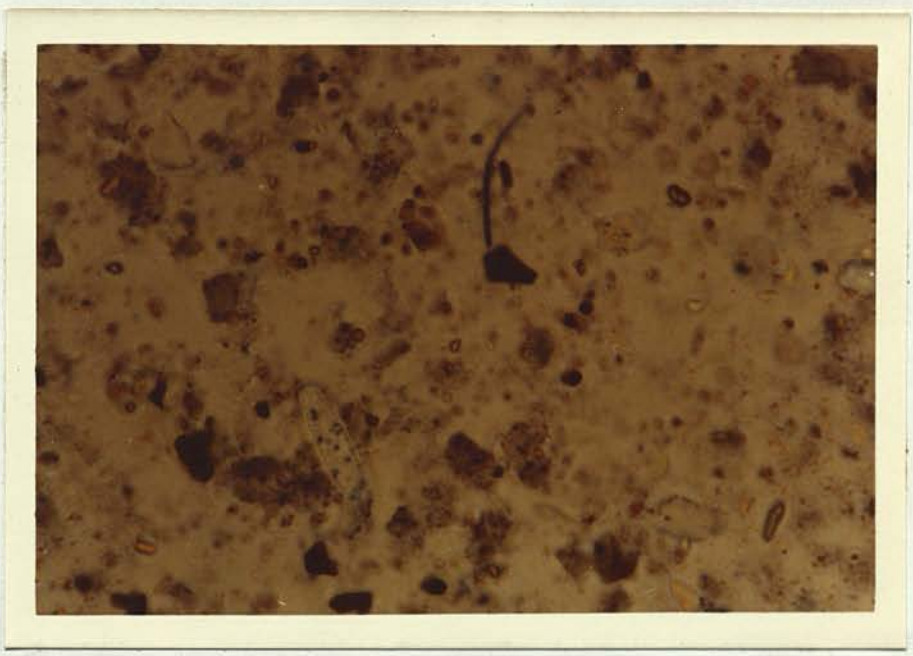


PLATE 2 ⊢ ≡ 10 µm

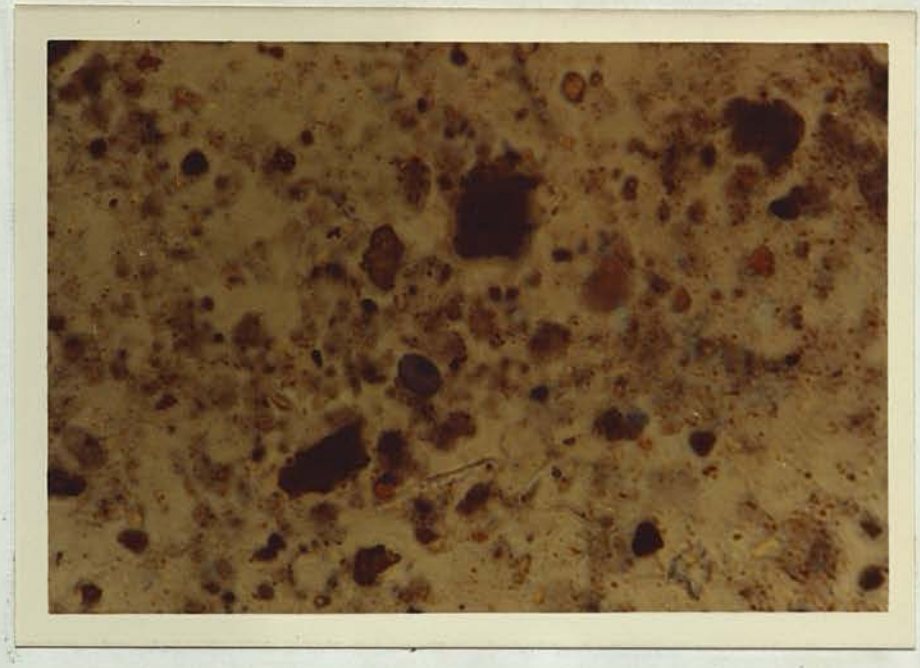


PLATE 3 ⊢⊣ ≡ 10 µm

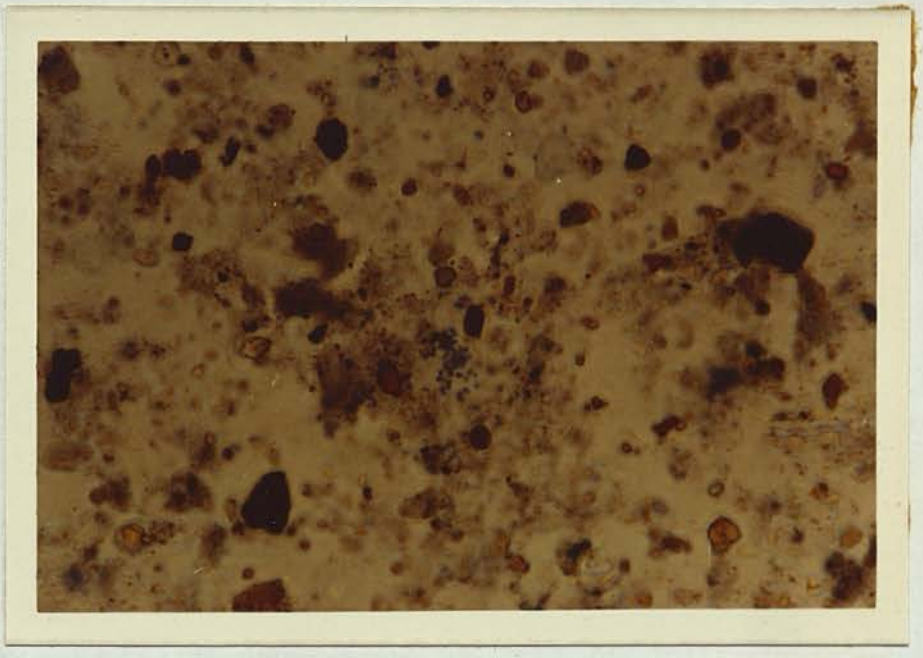


PLATE 4 ⊢⊣ ≡ 10 µm

PLATE 5 ⊢ = 10 µm

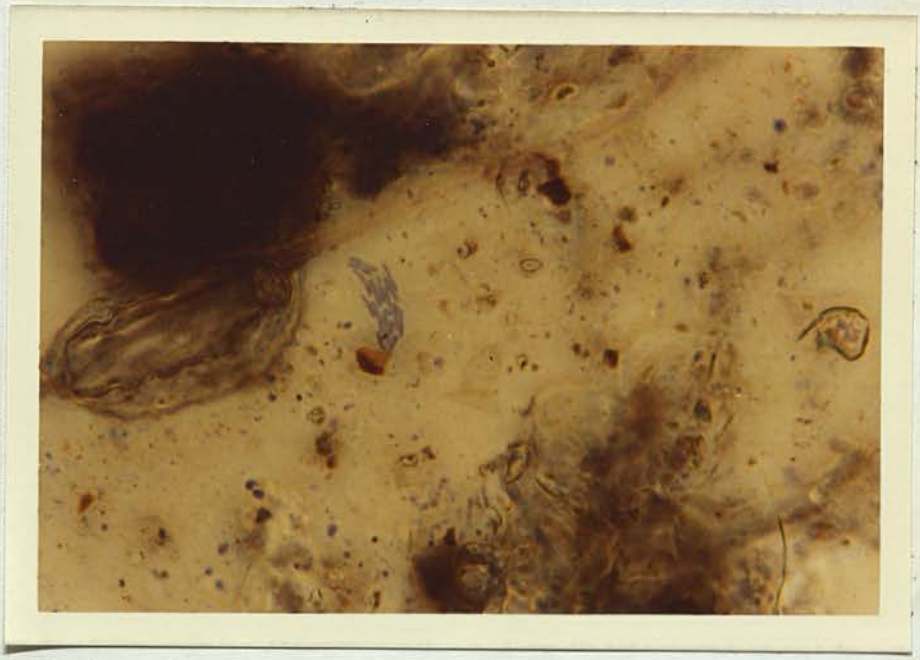


PLATE 6 ⊢ = 10 µm

B (2) Viable count methods

(a) Principles and problems

Although the shortcomings of viable count procedures in microbial ecology have been recognised since the inception of the method (e.g. Fisher, Thornton and Mackenzie, 1922), they have been largely ignored. Hence masses of almost meaningless data have accumulated in the literature; happily the (uncontrolled) specificity of the cultural methods of enumeration has recently received the emphasis it deserves - see for example Snyder, 1947; Stefanic, 1965; Jensen, 1967; Schmidt, 1973. The most important defect of cultural methods is the complete lack of information regarding their accuracy. This is inevitable since there is no alternative standard method to which cultural counts can be referred. It is also partly a matter of definition: the colony count is the sum of those organisms which when placed in synthetic nutrient surroundings, proliferate to produce colonies. This is of unknown (but probably little) ecological significance. As a criterion of competitive ability in a soil micro-environment, the ability of an organism to produce a colony on an agar plate is difficult to interpret.

In the absence of methods of assessing the accuracy of cultural counts, soil microbiologists have striven to increase and quantify their precision. Such a course of action is simple but the ease with which the precision of the method (and the absolute data obtained) may be altered by slight alteration of any of a number of experimental conditions is disturbing. The usual approach recommended (e.g. Parkinson 1970) has been to adjust conditions to give maximum counts. In examining one soil this is

satisfactory but difficulties are experienced in comparing different soils and possibly different microfloras since the methods used will not be the same.

The difficulties outlined above can be turned to advantage if the method is made completely selective; i.e. media and incubation conditions may be so adjusted to allow a single type of micro-organism to proliferate. However the preparation of an efficient selective medium is often difficult and examples of studies on soil in which a large variety of selective media have been used are conspicuous in the literature by their absence. For the reasons outlined above, the plate count as a method of enumeration was not used in this study. Before the decision not to use it was made, it was examined and the standard attempts to achieve maximal reproducible counts were made.

The nutrient status of a complex medium is selective in a largely unpredictable way as are the incubation conditions e.g. temperature, aerobiosis and the composition of the gaseous phase. Apart from these factors, the microflora being sampled also exerts a selective effect in that those organisms which grow fastest on a plate have a great competitive advantage and may prevent more delicate and slow-growing (and maybe more numerous) types from forming colonies. Consequently the use of any one medium and set of incubation conditions demonstrates the presence of only a(n) (unkown) proportion of the microflora. (According to Skinner, Jones and Mollison, (1952) this is between 0.1 and 1% of the total microscopic count). Thus a set of systematic errors caused by unsatisfactory cultural conditions accumulate. Random errors can

easily be introduced during sample dilution and plating but provided routine precautions are adopted, variations from this source can be reduced to an acceptable level. It is generally assumed that although the proportion of the microflora detected by the plate count is unknown, under similar conditions, the same technique will detect the same proportion. This is only likely to be true if quantitative changes in the microflora are unaccompanied by qualitative changes - in the terminology of Section A(p.63 et seq), if there is complete tight coupling between all community components and reproduction is concerted. It should also be noted that for the assumption to be true, all micro-organisms must have in soil, identical growth and death rates. These must be unlikely events. However, they were not fully appreciated initially and some effort was exerted towards obtaining maximal plate counts from soil samples.

(b) Dispersion techniques and cultural conditions

Examination of various dispersion techniques and the effects of different diluents proceeded much as described in p. 85 et seq for total count procedures and will not be reiterated. It was found that 10 minute mechanical shaking in calgon solution gave counts ca. 100% higher than when PBS or distilled water were used as diluent. Shaking also produced uniformly higher counts than blending or sonication. Counts were made on a soil extract yeast extract agar (SEYE):

0.2% Difco yeast extract

50% v/v soil extract (prepared as the filtrate from 1 kg soil + 2 litres of water autoclaved 1 hour, 120°C)

0.04% w/v K_2HPO_4

1.5% agar

Table 3. Increase in SEYE plate count with time. (18-20°C)

Sample	A	B	C	D	E	F	G	(garden soil)
1 week	129	197	151	134	195	135	176	mean x 10^{-5}/g dry soil
2 "	44	85	42	56	57	43	27	" x 10^{-6}
3 "	54	108	52	95	74	77	33	" x 10^{-6}

After 4 weeks incubation, an average increase of one colony per plate over that at three weeks was considered insignificant. Thus for maximal counts, samples were disaggregated by 10 minute mechanical shaking in calgon, plated on SEYE and incubated three weeks at ambient temperatures.

The nature of the medium chosen for plate counts appears to have changed gradually over the years from a simple partly or wholly defined medium (Thornton, 1922) to the opposite extreme of extremely rich complex media (e.g. Casida, 1965). When tryptone soya agar (TSA, Oxoid) (an example of the latter type of medium) was compared to SEYE, it was found that 0.3% TSA (with the agar complement made up with nutrient free agar) gave results quantitatively indistinguishable from SEYE when pour plate preparations were made. Greater concentrations of TSA were useless since plates were spoiled by the large spreading colonies of spore-forming bacteria. When surface-plating of inocula with a glass spreader was compared to pour plate procedures, it was found that an increase in count of ca. 100% was obtained. Whether this was a result of oxygen or heat sensitivity of the microflora was not investigated. The use of a proprietary medium possessed some advantage over the use of SEYE in terms of time saved and a supposed greater reproducibility.

(c) The one bacterium/one colony assumption

As normally used, the plate count procedure is based on the idealised assumptions that:-

(1) every viable bacterium on the plate forms a colony and

(2) each colony is formed as a result of growth initiated by one bacterium.

The first assumption was mentioned earlier and is virtually impossible to test rigorously. It is generally appreciated that its assumptions are violated. The probability of the second hypothesis being violated has also been recognised, but this more approachable problem appears only to have been investigated by Skinner, Jones and Mollison (1952).

Accordingly, four smears were prepared by the method described (p. 37) from a sample of garden soil. Disaggregation was carried out as it would have been for a plate count. A field of 1 cm^2 was delimited in the microscope eyepiece (the area subtended on the slide was not calculated). A significant number (>300) of potential colony forming units (PCFU) - i.e. groups of one or more bacteria, was counted on each smear. The total number of bacteria (T) in the PCFUs was accumulated simultaneously allowing an estimate of the T/PCFU ratio to be made. The results are shown in Table 4.

Table 4. T/PCFU in a garden soil

		PCFU	T	Subtotal ratios
Slide 1.				
fields	5	66	170	2.58
	10	130	336	2.58
	15	178	472	2.59
	20	236	643	2.72
Slide 2.				
fields	5	59	158	2.68
	10	119	333	2.72
	15	208	489	2.45
	20	213	710	3.33
Slide 3.				
fields	5	148	274	1.85
	10	257	559	2.17
	15	367	781	2.13
	20	474	1024	2.14
Slide 4.				
fields	5	162	302	1.88
	10	287	581	2.02
	15	414	814	1.96
	20	538	1056	1.96
Totals	80	1561	3433	2.30

Between five field variation was small per smear; between smear variation was larger. Overall variation was sufficiently small to justify the assumption that a valid estimate of the mean was obtained. With T/PCFU = 2.3 the results indicated that a plating procedure which allowed all living bacteria present to proliferate would underestimate the true viable count by 130% in this soil. (The results are very similar to those of Skinner et al, loc.cit.). This assumes that the distribution of bacteria within PCFUs is independent of their ability (or lack of it) to grow on a specified plating medium. Objection could be made to the conclusions above on the grounds that the dilutions of suspension used for smear preparation are lower than

those used for plate inocula. Thus further mixing of the dilutions would occur in sample preparation. However, such agitation is not designed to break PCFUs into single cells, but simply to disperse them randomly in the diluent. (If however PCFUs did break up, no constant relationship could be expected between serial dilutions. Such a lack of correlation is in fact often experienced.).

In this case, to obtain the maximum plate count, a correction factor of X 2.3 must be applied to the mean colony count. It appears therefore that the execution of a plate count on soil should be accompanied by microscopic examination to obtain a value for T/PCFU. Since a time-consuming microscopic examination is necessary, it would obviously be preferable if a microscopic estimate of viability could be made directly. If this were possible, the plate count could then be dispensed with and since the cells examined could be measured, the data could be more satisfactorily converted into biomass. Accordingly the plate count was ignored in this study and attention directed towards a microscopic demonstration of activity.

(d) The most probable number method for the enumeration of protozoa

Cultural methods involving the use of most probable number methods are widespread. They are based on the statistical method of maximum likelihood applied to replicated series of cultures made from a dilution series derived from the original sample. An end point occurs in the series at that dilution above which no microbial growth occurs. If this is a 1/n dilution, then micro-organisms were present in 1/n of the

original sample. Numbers of micro-organisms per unit of sample are usually estimated from the pattern of positive and negative replicate cultures at certain dilutions (e.g. the McCrady procedure for coliforms in water) or from the total number of negative cultures in a standard dilution series e.g. the method of Singh (1946) for protozoal numbers. Positive and negative cultures may be defined on the basis of presence or absence of unspecified growth or on the basis of some specified and recognisable activity e.g. nitrate reduction, denitrification, sulphide precipitation etc.

The probable importance of protozoa in soil microbial community dynamics has already been noted; hence it was of interest to assess the ease and reliability with which their numbers in soil may be estimated. All existing methods are highly selective (Heal, 1971); testates can be counted in Jones and Mollison preparations (see Plate 3) while most of the more delicate protozoa must be demonstrated culturally. This is usually done by modifications of a most probable number method developed by Cutler (1920) and Singh (1946). Although errors and confidence limits may be attached to estimates made in this way, the procedure is recognised to be imprecise and inaccurate, probably however no more so than the plate count for soil bacteria (Heal, pers. comm.). Since testates had occasionally been observed in Jones and Mollison preparations, an experiment was set up to assess the usefulness of the dilution culture method.

A sample of garden soil was homogenised for 30 seconds on a wrist-action shaker. The suspension was then incubated at room

temperature for four days. It was established by phase contrast microscopy that a bacterial bloom occurred in the first two days and that this was followed by protozoan bloom.

Petri-dishes containing 15 ml 0.5% Noble agar (Difco) + 1% NaCl were prepared. In each of these were placed eight autoclaved polythene rings (0.5 cm high) which had been cut from a length of tube 1 cm i.d. A thick suspension of Klebsiella aerogenes was smeared inside each ring. The suspension was prepared by growing a non-mucoid strain of the organism in Davies and Mingioli medium (Cruickshank 1965) to stationary phase. The cells were harvested by centrifugation and washed twice in PBS before use.

A series of fourteen doubling dilutions of the soil enrichment was prepared in PBS giving a range of 1/10 - 1/81,920. Each dilution was allocated a petri dish and 0.05 ml was placed in each of the eight rings. From the total number of negative rings (i.e. those showing no protozoal growth after incubation) and the statistical tables presented by Singh (1946) an estimate of the number of protozoa in the original enrichment could be obtained.

After three weeks incubation at ambient temperature (18-20^{o}C) clearly visible protozoan colonies as described by Heal (1971) and illustrated by Singh (1946) were conspicuous by their absence. However when samples were removed and examined microscopically a large variety of protozoa including flagellates, testate and naked amoebae, euglenoids and a variety of encysted forms were seen. No myxobacteria were observed although there

was a large number of cyst-like bodies. These were indistinguishable from spore-like bodies associated with a hyphomycete which grew in most of the rings. A diffuse end point (in terms of negative rings) was apparent but was not used to determine protozoan numbers.

The method as described was seen to suffer from a variety of disadvantages. Apart from its inherent lack of precision, it was tedious to perform and required long incubation. The fact that it fails to differentiate between trophic and cystic forms has long been recognised and control procedures can be carried out to obtain an independent estimate of the number of cysts present. The problem of migration of protozoa between adjacent rings is probably best approached by placing the rings in empty petri dishes and pipetting small quantities of agar separately into each one. Fungal overgrowth of cultures could probably be overcome by the use of appropriate inhibitors or antibiotics. Apart from its selectivity, perhaps the major problem in the use of this method is that soil cannot be sampled adequately since only mild homogenisation procedures are permissible when delicate protozoa are considered. It was concluded that in its present form the dilution culture method for protozoa was not satisfactory for routine use because of the difficulties noted above. There is however at present no alternative.

B (3) Discussion: The applicability of available techniques for enumeration

The nature of the subject of soil microbial community dynamics dictates to a large extent the applicability of techniques. At the same time, the availability of techniques limits progress in any experimental subject. Thus the most important property of a method of enumeration in this context is that it enumerates something which is important in, or is related to, what happens during community activity in soil micro-environments. Plate-count methods immediately fall under suspicion since the criterion defining the statistical population measured by this method is an artefact of the technique. It is extremely difficult to envisage a means of correlating colony count data with any other parameter of community behaviour e.g. respiration or substrate depletion. By suitable adjustment of the methodology, it is however possible to envisage a clear cut correlation between colony counts and community behaviour. Thus a highly selective culture system demonstrating quantitatively the presence of a specific type of micro-organism would be useful in determining the relationship between cell numbers and some physiological function for which the organism isolated was alone responsible. A hypothetical example which might be considered is an attempt to correlate variation in measured soil cellulase levels with variations in aerobic and anaerobic cellulolytic micro-organisms. The organisms are readily cultured selectively and the enzyme may be easily assayed in soil (Benefield, 1971). Thus it was concluded that the standard viable count procedure must be rejected from studies of community dynamics; the proviso should be added that (intentionally) selective plate counts may provide useful data in specific circumstances.

The two remaining most important properties of an applicable technique are (a) that it should be comparitively simple to carry out and should yield results relatively rapidly; and (b) that it should be 'open-ended' in the sense of being capable of modification, improvement and automation in the light of improvements in related techniques e.g. methods of removing micro-organisms from soil and of differentiating micro-organisms on an ecological basis.

Slide culture methods suffer from the same defects as any other cultural method of enumeration.i.e. selectivity of the culture medium and incubation conditions, and interaction between adjacent clones of cells. The latter problem would be exacerbated in slide cultures because clones of cells would be only a few microns apart if their concentration were to be high enough to enable counting. Thus metabolites could readily diffuse from one clone to another and interfere with colony growth. Also, the differential growth of clones would result in overgrowth of the slide by the more rapidly dividing cells before the slower cells had started to divide. These difficulties prevent the application of slide culture techniques to quantitative soil microbiology.

With the rejection of cultural enumeration methods, the criteria of acceptability can only be applied to microscopic methods. On the basis of statistical difficulties mentioned earlier (p.88) soil smear methods were rejected in favour of agar films. Thus the discussion is resolved into deciding how closely the Jones and Mollison (1948) technique conforms to the requirements outlined aboce. That it measures a parameter of ecological significance is certain; the parameter can be defined as the total number of micro-organisms present in a soil sample.

This is made up of (undefined) proportions of active and inactive cells which at the present time is the main failing of the method. However, much useful knowledge can be gained from data on the variations in the measured parameter even though detailed information concerning its composition is lacking. The method does however fall far short of the requirement that it be easy to carry out; in reality it needs meticulous care and patience to carry out and some experience to interpret. Its results can be obtained the same day if required which is perhaps as rapidly as may reasonably be expected. The most important advantage of the method is that being made up of a number of distinct experimental stages it is eminently suitable for development and improvement. For example, the final discriminating and enumerating stage is probably the most taxing to the experimenter. With the availability of logic circuitry, image-analysing computers and microscopic television photography there is no reason why this stage in the analysis should not be almost completely automated.

Thus it appears that despite its shortcomings, the agar film microscopic enumeration method possesses certain advantages over all other methods considered. It was consequently adopted for use in this study and attention was directed towards eliminating its most obvious defect i.e. its lack of discrimination between active and inactive cells.

(C) The detection of active micro-organisms in soil

(1) Principles

An unresolved attempt to estimate the total number of actively dividing cells in soil samples has already been described (Section A). The present series of experiments was based on the possibility of carrying out a cytochemical analysis on cells in an agar film. Thus if the term 'active' is interpreted as 'possessing specific enzyme activity', techniques can be designed to differentiate active and inactive cells on a visual basis. The ecological significance of the differential criterion must first be considered. Initially no differentiation was desired of different taxonomic types of micro-organism, thus an enzyme function shared by all or the majority of micro-organisms was sought. At first sight, the known metabolic diversity of micro-organisms makes this appear a daunting task. However, according to Kluyver (1953) microbial metabolism does conform to a definitely recognisable pattern. Microbial transformations of nutrient materials follow two main sets of biochemical pathways termed anabolic and catabolic processes. Both anabolic and catabolic processes can be reduced to chains of elementary reactions of a very restricted number of types and these are similar in all organisms. In terms of anabolism, there is remarkable uniformity in the end-products and this is largely independent of the nature of primary nutrients and the taxonomic status of the organism. Catabolic processes are characterised by excretion of the end products which may comprise a varied list such as CO_2, N_2, acetic acid or ethanol in different organisms. Nonetheless all these catabolic products have resulted from intracellular oxidation-reduction reactions which differed only in

the nature of the reactants and the products. Thus although it is the catabolic processes which differentiate various micro-organisms in terms of end products, all organisms which by any reasonable definition may be termed active must be carrying out these energy-yielding processes.

It should be noted that this loose definition of activity does not stipulate that the cell should be growing (i.e. exhibiting anabolic activity). In the past the interpretation of such terms as 'living', 'viable', 'active' with respect to soil micro-organisms has often been coloured by an imagined relationship between these properties and the ability to divide and procreate - more specifically, to be able to produce a colony on an agar plate. It must be asked whether the interpretation of an active cell as one capable of multiplication (or in the process of multiplication) is strictly applicable to the function of a microbial community in soil. This function has been defined (p. 1) in terms of substrate transformation. Although little information is available on the proportions of substrate transformation in soil which result from anabolic and catabolic processes, it can be seen that a community is theoretically capable of at least restricted function in the absence of anabolic function in some of its components. The mandatory linkage between anabolism and catabolism in the cells metabolism means that for most of the time, community function will be a result of both processes. What little information is available (McGill, Paul, Shields and Lowe, 1973) suggests that a carbon assimilation efficiency of ca. 60% occurs in soil systems. This is in accordance with results reported for organisms in aqueous cultures

(Payne, 1970). This indicates that 60% of substrate carbon is immobilised in microbial tissue; if continued indefinitely, this accumulation of carbon would result in immobilisation to the detriment of the ecosystem. Clearly this does not occur; in a climax community the level of soil organic matter is relatively constant. Thus all organic matter entering the soil over a suitable time period must leave ultimately as products of microbial metabolism. (This is in reality a physiological formulation of the necessity for periodic senescence or predation in a microbial community.).

In this way, a sound case may be made for the adoption of 'exhibition of catabolic activity' as an ecologically meaningful parameter in the study of soil microbial communities. It is inferred that catabolic activity will normally be accompanied by anabolic activity, but at certain stages in the microbial population growth cycles this is not necessarily so. The question then arises as to whether such an activity parameter is to be a qualitative or quantitative one. Ideally an assay method could be envisaged which made a clear distinction between catabolically active and inactive cells. This is not however realistic since various levels of activity are to be expected depending on whether catabolism is accompanied by anabolism and more directly on the nutrient status of the cell. Thus although all catabolically active cells may contribute to the function of the community, the assessment of the lower limit of activity is an arbitrary one governed probably primarily by limitations of the assay method being used and secondarily by the requirements of the experiment being carried out.

The over-riding similarity of catabolic processes in different micro-organisms is a result of the same basic types of reaction (i.e. oxidation-reduction) being used for the purpose of harvesting energy from diverse substrates. These reactions are mediated by the enzymes called dehydrogenases; the essential similarity of these systems in most bacteria is manifested in terms of a restricted group of co-enzymes which act as intermediate electron carriers in a wide variety of organisms. Thus electron carriers at early stages in the electron transport systems frequently take the form of pyridinoproteins (co-factors NAD and NADP); electrons may be passed from such carriers to flavoproteins (co-factors FAD and FMN) and ultimately via cytochromes to oxygen in respiration or more directly to some oxidised organic material in fermentation.

The problem which these facts posed in the present context was visualised as the provision of a reagent containing a suitable artificial electron acceptor whose reduction would create a visually recognisable difference between catabolically active and inactive cells. The use of artificial electron acceptors which in the reduced form are coloured is routine in some branches of biochemistry; their reaction is, however, usually assessed at the macroscopic level. The difficulties envisaged in the application of such a technique to agar films containing soil suspensions were those of pigment quality and reaction specificity. These problems are shared to some extent by studies of the enzyme histochemistry, and more particularly cytochemistry, of animal and plant cell preparations. Consequently some of the methods used in these subjects were tried with appropriate modifications on preparations of soil suspensions.

C (2) Trials of artificial electron acceptors

(a) Types of compound available.

Klett (1900), used potassium tellurite to examine cultures macroscopically and noted that some cultures accumulated a black pigmentation of metallic tellurium. Lakon (1939) attributed this reduction to dehydrogenase activity. Wachstein, (1949) demonstrated that a variety of species of Salmonella, Shigella, Proteus, Pseudomonas, Bacillus, Staphylococcus, Corynebacterium and Clostridium accumulated granular deposits of tellurium when incubated with tellurite at pH 7.4 and compared this with the use of tetrazolium salts for cytochemical demonstration of dehydrogenase activity. Tellurite is less sensitive than tetrazolium salts for demonstrating dehydrogenase activity in tissue sections (Gomori, 1952). The tellurite method has however discriminated between colonies of normal yeasts and respiration-deficient mutants (Nagai, 1965).

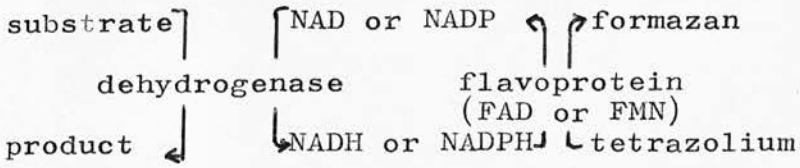
Redox dyes including methylene blue have been employed to demonstrate dehydrogenase activity in tissue sections (Burstone, 1962); many however exhibit properties the opposite of those required in this study i.e. the oxidised form is coloured and the reduced form is colourless. The best electron acceptor in cytochemical studies is the tetrazolium salt. These compounds possess a heterocyclic ring containing one carbon and four nitrogen atoms, one of which is quaternary. Tetrazolium salts are colourless

or pale and are readily reduced to form intensely coloured water-insoluble formazans. A continuously increasing range of tetrazolium salts is available, the compounds varying with regard to the number of heterocyclic rings in the molecule and the nature of the (often aromatic) substituents on the heterocyclic rings. Tetrazolium salts are mostly photosensitive as are many of the formazans. This was envisaged as a problem when microscopic examination of films was considered and restricted the choice of possibly useful acceptors. Triphenyl tetrazolium chloride (TTC) is used to detect dehydrogenase activity on a macroscopic scale in soil (e.g. Casida et al, 1964; Lenhard, 1966; Klein et al, 1971). After incubation of soil samples with TTC, the formazan is extracted by an organic solvent and assayed colourimetrically.

In both the macroscopic TTC method and the cytochemical method under development, no energy-yielding substrate is used in the reaction mixture. Thus the amount of formazan produced is related to dehydrogenase activity occuring in the soil at the expense of natural substrates. If extraneous substrates were used, formazan concentration would be a function of enzyme concentration rather than activity.

Mode of action of tetrazolium salts

Cascarano and Zweifach (1959) proposed the following general scheme of electron transfer to tetrazolium salts:



In natural electron transport systems the flavoprotein would transfer electrons to the next carrier molecule in the chain. The term diaphorase has been applied indiscriminately to any enzyme capable of catalysing the oxidation of NADH or NADPH by any of a variety of artificial electron acceptors (Mahler and Cordes, 1967). Since many flavoproteins are capable of being reduced by NAD(P)H and practically all reduced flavoproteins are capable of passing electrons to artificial acceptors, the term 'diaphorase' is now considered to have little physiological meaning and is falling into disuse. Many cases of tetrazolium reduction in the histochemical literature have been ascribed to diaphorase activity; thus the specificity of the acceptor has in some cases been overemphasised. In this study it was judged to be an adequate method of demonstrating the presence of functional components of the electron transport system.

(c) Preliminary tests of tellurite and tetrazolium salts on bacteria.

During the study of a newly synthssised tetrazolium salt (nitroblue tetrazolium, Tsou et al, 1956) Nachlas et al (1957) provided a list of properties which should be possessed by the ideal tetrazolium salt:

(1) It should be readily reduced to the formazan and should resist oxidation by molecular oxygen back to the tetrazole.

(2) It should have a strong attraction for the electrons liberated by the substrate so that atmospheric oxygen does not act as a significant competitor.

(3) The formazan produced should have good pigment quality and should appear in the tissue as an amorphous pigment rather than as crystals.

(4) The formazan produced should have very low solubility in aqueous solution and in the common organic solvents and should bind to protein and be insoluble in lipids.

For the present purposes the formazan should not be photolabile. These requirements are most closely met by the p-nitrophenyl substituted ditetrazolium, nitroblue tetrazolium (NBT). This compound was tested along with tellurite, TTC and the ditetrazolium, blue tetrazolium chloride (BT).

Method

The three tetrazolium salts were made up separately to 0.02% in 0.1M phosphate buffer pH 7.4; tellurite was made up to 1% in the same buffer. Five colonies were taken at random from a dilution plate prepared from garden soil and were suspended in water. A drop of cell suspension was mixed with a drop of acceptor solution on a micr-

oscope slide. Cover slips were placed on top of the preparations which were examined at 30 minute intervals.

Results

Long reddish crystals of tellurium were clearly visible after several hours in the larger cells. In small cells these were however difficult to distinguish. Of the tetrazolium salts, only TTC showed appreciable colour formation. The red monoformazan produced was however laid down extracellularly as well as inside the cells. This may have been caused by photolability of the tetrazolium. Because of the non-uniform staining of cells, interpretation of the appearance of stained cells was subjective. Thus improvements in technique leading to intensification of staining could only be judged by eye and could not be readily quantitated.

(d) Modifications of the staining method and the introduction of control inactive cell preparations.

Since NBT conformed most closely to the requirements listed on p. 118 , attention was concentrated on this compound as the optimal reagent. Burstone's (1962) staining procedure was used on cell suspensions prepared as above. This gave excellent staining - black individual cells which were blue in the mass. (See Plates 7, 8 and 9). Similar localised staining has been noted in several species of Clostridium by Davis and Mudd (1957) who used TTC, BT, tellurite, Janus green B and neotetrazolium chloride.

Methods

As noted above, small cells presented interpretative difficulties; consequently samples of OX1 (Section A), a small organism, were examined. The effects of NBT on a mixed population derived from soil and stained in an agar film were also investigated. 5 mg NBT was dissolved in 0.25 ml absolute ethanol and mixed with 30ml distilled water with constant agitation. The solution was made up to 90ml by the addition of 0.2M tris-HCl buffer (pH 7.4). Agar film preparations were made of OX1 and soil suspensions by the method described earlier (p. 89 et seq). After drying, these films were immersed in the staining solution and were examined after 3, 5 and 12 hours incubation at room temperature. Films were also prepared from pasteurised OX1 (70^{o} C, 15 min), boiled (1 min) soil suspension and formalin-treated soil suspension. For the latter control, suspensions were made up to 10% v/v formalin and held for 10 min after which they were centrifuged, washed and resuspended in water prior to use.

Results

Extended incubation was necessay for adequate visualisation of formazan in agar films. Staining was faint in 5 hours and significantly more intense after overnight incubation. None of the control de-activated preparations showed staining in 3-5 hours. However, after overnight incubation, the boiled and formalin-treated soil prepar-

ations contained motile, well-stained bacilli. These were presumed to have arisen from spores which survived the deactivation procedures.

(e) The use of counterstains and optical filters.

5% ethanolic erythrosin and the following dyes (2% aqueous) were applied to soil suspensions which had been incubated 5 hours with NBT in buffer: congo red, basic fuchsin, safranin O, eosin W.S. , acridine orange and methyl orange. The only dye which brought about a reasonable degree of contrast between formazan deposits and the rest of the cell was safranin. Clearer demarcation was obtained when sodium succinate (15 mg/ml) was added in the distilled water used to prepare the NBT. The effect of susbstrate addition was not unexpected and although probably useful in some circumstances, this procedure conferred an undesired specificity on the method.

Kodak 'Wratten' gelatin filters, colours 12 and 22 (yellow and orange respectively) were prepared as discs and inserted in the light path of the microscope below the condenser. Both colours were useful in increasing contrast between formazan and unstained cytoplasm, 12 being superior to 22. The use of filter 12 was adopted routinely.

(f) The use of substrates, co-factors and intermediate electron acceptors.

Since succinate increased the intensity of staining, the

level of catabolic activity of cells in soil was probably too low to permit maximal formazan deposition. Although staining was obvious with NBT and more obvious with NBT + succinate, it was not considered justified to try to evaluate any difference in number of cells stained by the two procedures.

The following compounds were tested as potential energy-yielding substrates: L-glutamate, DL-alanine, glucose-6-phosphate, glucose, malate, ethanol, α-ketoglutarate, DL-isocitrate and lactate. The effects of these substances on soil suspensions in agar were compared with the dehydrogenase activity taking place as a result of endogenous substrates and demonstrated by the use of buffered NBT without substrtate. The pH of the buffered NBT used with potential substrates was modified to suit the optimal range of well known examples of the primary substrate dehydrogenases (Mahler and Cordes, 1967). Only in the case of glutamate was there any significant intensification of staining. The terminal concentrations of the substrates in this experiment was 50 μM. Addition of the co-factor NAD to glutamate-NBT and FAD to succinate-NBT at the same concentrations did not exert any obvious effect. This was not unexpected in the latter case since the flavin part of succinate dehydrogenase is firmly bound to the apoprotein. Use of reduced co-factors along the lines of the classical diaphorase assays was not consid-

ered warranted since it was observed that addition of NADH to a solution of NBT gradually induced precipitation of the formazan.

A method of increasing the extent of reduction of artificial electron acceptors which is commonly used in histochemistry and biochemistry is the insertion of an intermediate electron carrier between the source of reducing power and the synthetic oxidant. The use of phenazine methosulphate (PMS) in a 6:1 molar ratio with NBT was investigated on soil suspensions in agar films. Use of the compound with NBT, either alone or supplemented by succinate or glutamate did not result in any increase in rapidity or intensity of staining. Neither did anaerobic incubation improve staining, a finding in agreement with the original description of the application of NBT (Nachlas et al, 1957).

(g) Fixation and prevention of multiplication.

The problems envisaged in the use of the method were (1) that of finding some means of improving the sensitivity of the tetrazolium reduction procedure and (2) that of preventing multiplication (or increase in activity) during incubation.

Altmann and Chayen (1965) found that when unfixed cryostat sections of rat liver were incubated with tetrazolium salts

for the demonstration of pentose shunt dehydrogenases, the electron transport system must be intact and functional. It is possible that during the reaction of bacteria in soil with NBT, some components of the electron transport system are lost from the cells thus decreasing the maximum amount of formazan deposition possible. The relatively increased importance of metabolic losses from cells growing at low rates has already been noted (p.48). It has been shown that in some bacteria at least, electron transport components and some dehydrogenases may be considered as membrane markers (Salton, 1971). With the membrane of Micrococcus lysodeikticus, Ellar, Munoz and Salton (1970) demonstrated that NADH dehydrogenase may be removed by washing in buffer. Release was prevented when the membranes were treated with glutaraldehyde. Glutaraldehyde acts as a cross-linking agent between the amino functions of proteins (Bowes and Cater, 1965; Flitney, 1965); protein conformation is preserved and membrane-bound enzymes may be firmly fixed in position. Under suitable conditions of glutaraldehyde concentration and length of treatment, no adverse effects appear to be exerted on the activity of dehydrogenases (Ellar et al) 1970).

1ml of a soil suspension was mixed with 1ml of 1% v/v glutaraldehyde in 0.1M phosphate buffer pH7. The mixture was immediately centriguged for 2 min in a 'Quickfit' micro-centrifuge. The supernatant was removed and the pellet washed before being sedimented and resuspended in

water prior to standard NBT staining. This procedure resulted in an improvement in the rapidity of staining; the ultimate intensity also appeared to be slightly improved. Good staining was obvious after four hours in buffered NBT but it did not become maximally intense until about eight hours. Staining periods of this length almost certainly allowed proliferation in the agar films. After overnight exposure to the stain microcolonies and motile bacteria were abundant. A means of sterilizing the films without affecting their enzyme activity was required. Exposure of the dried film to a dose of $1{,}35 \times 10^{4}$ ergs/mm^{2} ultra-violet radiation proved satisfactory in this respect. No cells could be cultured in tryptone soya broth from films thus treated when the cultures and the films were incubated in the light.

C (3) Application of the method to garden soil.

For quantitative estimates of the number of catabolically active cells in a soil sample, it was desirable that samples should be prepared in the way described for Jones and Mollison films. After NBT treatment, a 'catabolically active count' could be made. The same film could then be stained with PAB and a total cell count carried out. In this way, errors due to manipulative and distributional defects should be very similar in each count. This necessitated a slight alteration in the mounting procedure. Squares (2cm) of glass were cut from microscope slides; two such squares were glued to an intact slide leaving a central trough over which a coverslip bearing an NBT treated film could be fixed face-downwards. When examination was completed, PAB was injected into the space between the slide and coverslip.

This procedure was satisfactory with garden (mineral) soil samples; some examples of the effects of NBT staining are provided in Plates 7, 8 and 9. It was concluded that the method could be applied satisfactorily to mineral soils for the purpose intended. When however it was tried on the model soil described later (p. 128) it was not satisfactory. The model soil was derived from the A horizon of a podzol and contained 16% organic matter.[1] Much of the organic material was in a finely comminuted form and being humified, appeared dark microscopically.

Discrimination between such small organic particles and bacteria containing formazan was inadequate for quantitative work. Although the procedure was valid and presented a very powerful new technique in soil microbiology, it could not be applied in the present study. It is possible that the sensitivity of the method could be improved by the use of a better tetrazolium salt. This was not investigated further.

Legend Plates 7, 8 and 9

NBT-formazan labelling takes three forms:

(1) Total or partial cytoplasmic membrane staining. (Plate 9, 1, 2, 3, 4 and 5).

(2) Terminal or polar accentuation of staining (Plate 8, 1; Plate 7, 1, 2; Plate 9, 6.).

(3) Membrane and presumed mesosome-type organellar staining (Plate 8,2; Plate 7,2).

The mode of staining of small organisms is problematical since the whole organism appears to be stained. (Plate 8,3; Plate 7,3; Plate 9,7.).

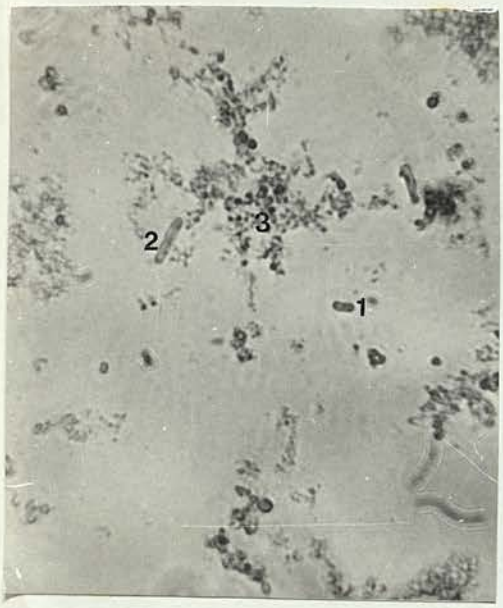


PLATE 7 c.450X

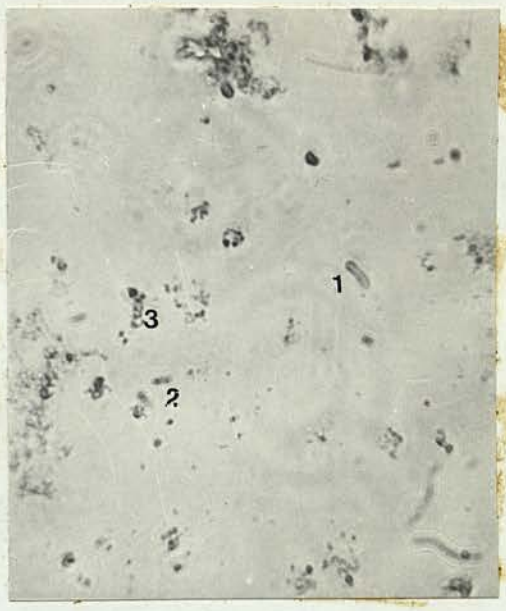


PLATE 8 c.450X

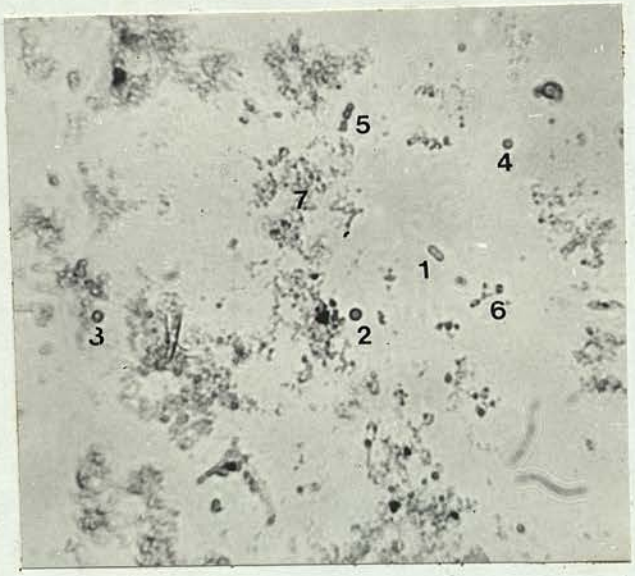


PLATE 9 c.450X

SECTION D:

The preparation and properties of a model soil

D (1) Preparation

A basic problem in any approach to the study of soil microbial community dynamics has been formulated (p. 79) as: "What sort of soil samples should be studied?" Two basic types of answer to this question are in currency among soil microbiologists engaged on quantitative problems. Firstly, a natural soil may be studied, samples being removed to the laboratory for analysis with as little disturbance as possible. Changes in parameters of the microflora are measured along with uncontrolled natural variations in environmental parameters thought to be of some importance. This was the approach of Cutler, Crump and Sandon (1922) in their monumental year-long experiment. For the effort exerted in such an approach the rewards are scant in terms of correlation between variations in microflora and environmental parameters. Hence the second basic approach may be considered. This involves the creation in the laboratory of an idealised soil whose properties are very well known. The soil can be made as homogeneous as desired and can be studied under controlled conditions where the environmental parameters may be varied as independently as is physically possible. The second type of approach was adopted in this study. The nature of the experiments envisaged governed to a large extent the chosen properties of the model soil. Model soils made from glass beads (Parr and Norman, 1964), sand or ion-exchange resins have been used, but in this study it was decided to derive the model soil from a real soil. Thus the properties of the model soil are intimately connected with those of the real soil. It was decided

that (1) the model soil should be free-draining to allow the addition of aqueous solutions and the maintenance of some degree of homogeneous aeration status; (2) it should have a high C/N ratio so that on amelioration of the limiting conditions a clear-cut growth response could be expected; (3) the model soil should be sufficiently well characterised and its parent natural soil should be of sufficiently widespread occurrence that the model could be reproduced by investigators in other laboratories.

The real soil should be natural, unamended and possess minimal spatial variability. It should preferably support a climax community canopy. From the sampling point of view, it should possess horizons which are clearly defined visually, physically and chemically to allow for reproducibility and the choice between a variety of potential models. Information should be available on its pedology, its physical and chemical properties, its micro-morphology and microbiological status.

Local soils were considered and it was decided that a natural podzol which had been suitably characterised and analysed would fulfil most of the requirements. The sampling site chosen was one which had been surveyed by the Scottish Soil Survey and lay at 535 m (1750 ft) above sea level in a natural ride (ca. 50 m wide) between adjacent Forestry Commission plantations. The vegetation was representative of that present before plantation with conifers and was continuous with an area of natural vegetation outside the plantations. Since microbiologists rarely receive formal tuition in soil science it is valuable to elaborate some of the basic details about podzols and podzolisation. This has been

done in Appendix A.

D (2) Description and sampling of the chosen soil

The nature of the soil is described in detail in Appendix .

A flat area close to the site described was chosen as the sampling area in the present study. Four pegs, mutually ca. 2m apart were placed in the ground and vegetation recorded in 1m quadrats around them. Pits ca. 0.75 m deep were excavated round the pegs and samples of the H, A, and B_1 horizons taken from each face of the pits. The samples were pooled to make one sample per horizon per pit. On return to the laboratory, each sample of each horizon was rubbed through a 3 mm sieve and bulked, thus obtaining one sample per horizon. The vegetation found was as described on p. 210 with Calluna dominant and occasional Sphagnum spp present. Profiles noted in the pits were compatible with the example on p. 212 with the exception that the B_1 horizon was erratic in thickness and occurrence, sometimes being present on one side of a pit and absent from the opposite side. Since the B_1 horizon was extremely difficult to sample in an uncontaminated form and the A_o(H) was obviously dominated by fungi, sieved A_1 was taken as the starting point in the preparation of the model soil.

D (3) Moisture characteristics of sieved A_1

The importance of the drainage properties of the model soil has already been mentioned (p.129). The moisture characteristic curve of the soil was determined to allow estimation of the pore size frequency distribution. From this information it is possible to predict the water-filled pore space at various gravimetric moisture contents.

Method

Gravimetric moisture content was calculated on a dry weight basis after drying samples to constant weight at 105°C. This was carried out on 10 g samples which had been saturated with de-aerated water for ca. 24 hours prior to equilibration over a range of pF. pF < 100 cm were established on a tension table; higher suctions were provided by pressure membrane systems. (15 bar ceramic membrane for pF 4.2; 3 bar for pF 3.5 and 3). Correlation between various suction parameters and the diameters of drained pores are assembled in Table 5 (compiled from a variety of sources).

Table 5. Correlation of suction parameters.

cm of water	atmos-pheres	$lb.in^{-2}$	milli-bars	pF	R.H.	A_w	diameter(μm) of pores drained
10		0.14	9.81	1.00	100	1	296.6
103	0.1	1.46	101	2.01	100	1	29.8
1033	1	14.7	1013	3.01	99.9	0.999	2.87
10330	10	146.9	10130	4.01	99.1	0.991	0.29
15495	15	220.4	15200	4.19	99	0.99	0.19
				5.17	90	0.9	
				6.00	50	0.5	

(I am indebted to Mr K Shali for the compilation of much of the information in this table).

Six replicate samples were prepared for each pF value and the mean gravimetric moisture contents plotted in Figure 14. The maximum water-filled pore size over a range of moisture contents was derived from Figure 14 and an extension of the table above; a cumulative curve of these values is shown in Figure 15. (The volume on the ordinate corresponds to that in all pores smaller than the values specified on the abcissa.). From this curve, the volume of water contained in a continuous series of 20 μm diameter ranges of pore size was obtained and plotted as a histogram (Figure 15).

Discussion

It can be seen from Figure 15 that the group of pores which make up the greatest proportion of the space in the soil is in the 0-20 μm diameter range. In terms of micro-habitats, not all of these are available to bacteria because of their size, and those which are can support only small colonies. When the information in Figures 14 and 15 is considered in terms of field moisture contents, some interesting conclusions may be drawn about microbial growth in this soil. A field moisture tension of ca. 100 cm is often taken as a reasonable average for a variety of soils. This is equivalent to pF 2.01 and moisture content of ca. 94% which falls within the range experienced in field samples. At this pF, all pores of diameter >29.8 μm are drained. The average volume of one bacterial cell in this soil has been determined as 4.5 μm^3 (p. 144). The volume occluded by ideally spherical bacteria depends on the shape of the container, but in general, it is fair to assume that if open or cubic packed, they will occlude a total space of twice their own volume. On

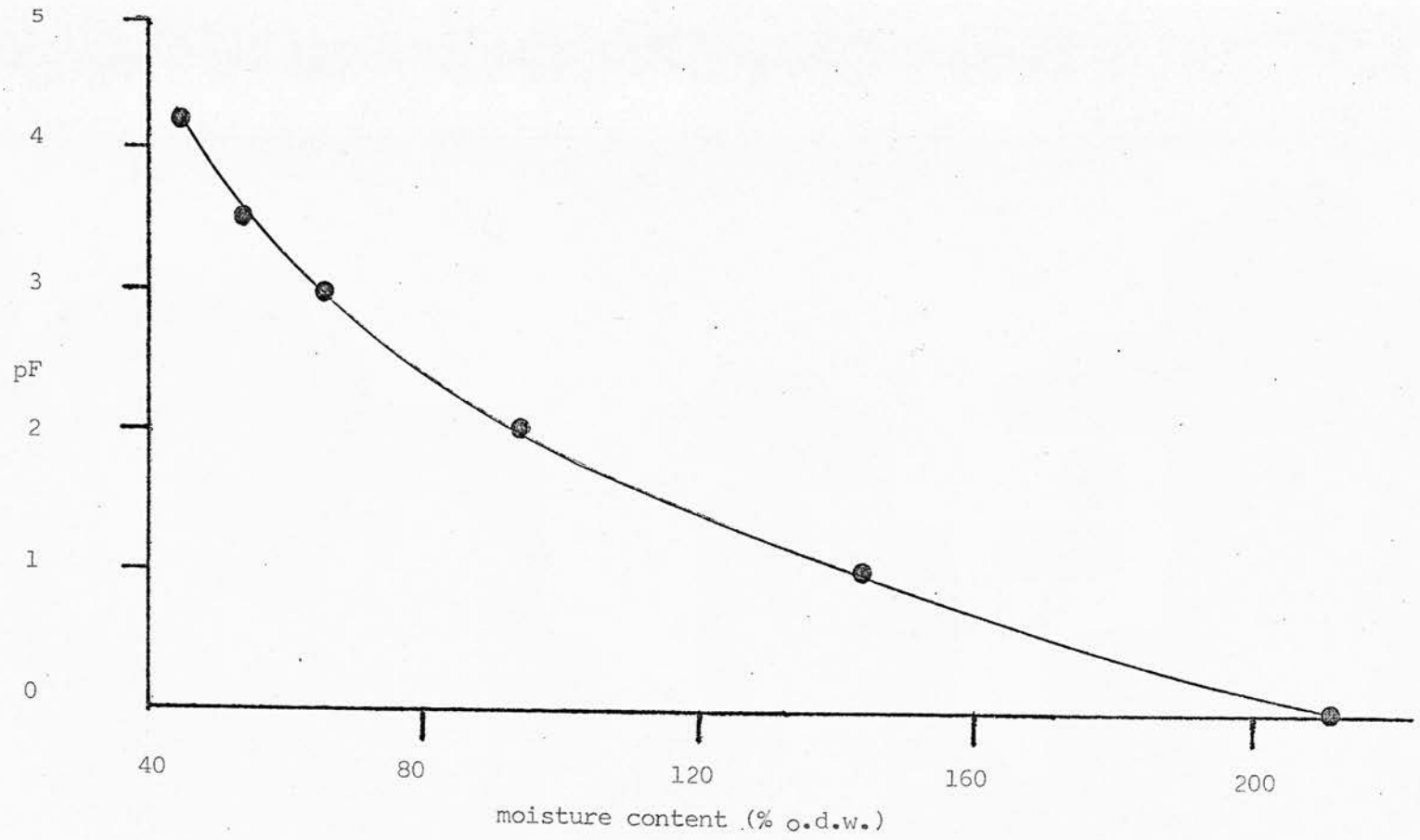


Figure 14. pF characteristic of the model soil

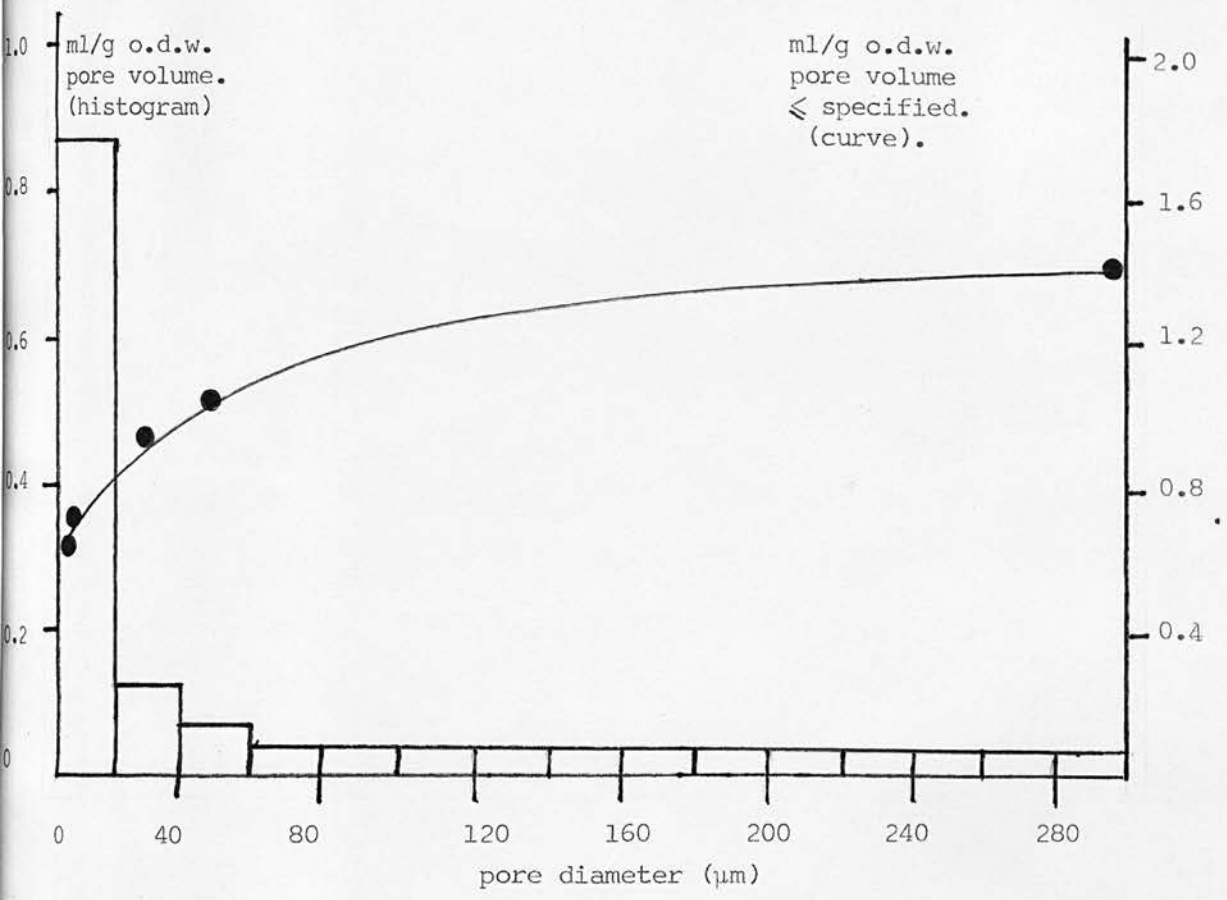


Figure 15. Pore-size frequency distribution in the model soil

this basis, the minimum pore volume which contains one cell would be 9 μm^3. If this spherical volume is considered to be situated in an equidiametrical cylindrical pore, the pore diameter is

$$d = 2r = 2\sqrt[3]{9/(4/3\pi.)} = 2.58\ \mu m$$

Thus at 100 cm tension, pores between 29.8 and 2.58 µm diameter are moisture-filled and large enough to contain bacteria. (Although this approximation probably overestimates the minimum occupiable pore size this effect is counter-balanced by the fact that the mean cell volume is probably an underestimate because of cell shrinkage in sample preparation.). From Figure 15 the total volume per g soil represented by this range of pore size is derived as 0.29 ml. Theoretically this could accommodate a maximum of 0.145 ml of cells, i.e. a total number of

$$(0.145/4.5) \times 10^{12} = 3.2 \times 10^{10}/g$$

In other words, moisture-filled pore space in excess of that which may be already occupied by bacteria, is theoretically available for colonisation by a maximal 3.2×10^{10} bacteria in each gram of soil. A total count determined by the Jones and Mollison technique on soil fresh from the field gave a standing crop estimate of $1.88 \times 10^9/g$, a figure which may be regarded as reasonable in comparison with other soils, and the same order of magnitude as counts found later in the same soil (p. 142 et seq). No information may be derived from this analysis on the whereabouts of bacteria i.e. whether they are in water or air-filled pores. If however, the not unreasonable assumption is made that active multiplication is restricted to water-filled pores, it is obviously possible for a 20-fold increase in cell numbers to take

place if water-filled pore space were the only limiting factor. Growth must therefore be restricted by forces within the microbial community (i.e. interactions among the component populations) or nutrient limitation in this soil at 100 cm tension. In either case it must be assumed that competitive interactions are vital to the persistence of specific types of micro-organisms in the soil. Since many interactions between microbial populations have been shown to result in periodic biomass changes (p. 44) it would not be surprising if community growth and activity were found to be oscillatory. Periodic growth has already been noted in many soils (p. 38) and periodic activity was later noted in this study (p.141).

The forces which may act within the microbial community to restrict proliferation are difficult to predict because of their variety. However, the observations of Nikitin (p.24) indicate that functional units of microbial tissue in soil consist of micro-colonies composed of some hundreds of cells. Thus the form and arrangement of micro-organisms in soil may provide one mechanism of community growth limitation. The space required by colonies of this size would restrict the "colonisable" pore-volume as calculated above by a considerable amount. Without some knowledge of the geometry of micro-colonies and their distribution it is impossible to estimate by how much this would be reduced. It is safe however to assume that the number of cells which could be accommodated in the manner indicated by Nikitin (1973) would be of a similar or the same order of magnitude as the number already present in the soil. Thus proliferation may possibly be restricted because of spatial

limitation especially if different surfaces were of varied acceptability to colonising micro-organisms. If this were the case, proliferation of specific portions of the microflora would probably be accompanied by a decline in the numbers of other portions. Provided each portion was to avoid extinction, periods of proliferation would alternate with periods of decline. Thus the probability of oscillatory behaviour is again indicated.

SECTION E;

Model soil microflora growth as a result of a stimulus at one point in time

E (1) Nature of the growth-promoting stimulus

If the growth and activity of the microflora in a number of replicate soil samples is to be followed it is important to be certain that activity is initiated simultaneously in all the samples. If for example growth and activity were going on in all the samples before the start of the experiment it would be safe to assume that the samples would be temporally heterogeneous i.e. activity in one sample would be unlikely to be in phase with that in another. Thus it is important that activity should be synchronised at the start of the experiment by the application of a growth-promoting stimulus which has such over-riding effects that it modifies the environment of the micro-organisms, resulting in very similar conditions in each sample.

Suitably sieved (3 mm) and mixed samples of the podzol A_1 horizon described (p.208-212) were adopted as the model soil in this study. The pH of this soil was 3.8; although this would allow fungal growth, it could be expected to exert a selectively deleterious effect on bacterial development. By altering the pH of the soil to a level which would allow bacteria to develop preferentially, a growth-promoting stimulus of the type outlined above could be simply applied to the soil. This treatment may be expected to exert a number of effects on the soil apart from simply altering the pH. These could include changes in the dissolved concentrations of ions, changes in charged surface hydration and ion-saturation, and changes in soil solution composition in qualitative and quantitative terms with respect to organic materials.

The selection of a model bacterial community in preference to a fungal/bacterial system was also made on practical grounds since the three dimensional dynamics of fungal growth (Marshall and Alexander, 1960; Mandels, 1965) are less amenable to analysis than those of bacteria.

For the purposes of the experiment, soil was incubated in 10 ml plastic disposable syringes. These held ca. 5 g (wet weight) of soil equivalent to ca. 3 g dry matter when filled to the 10 ml mark and tapped gently to eliminate air-pockets. Initially the soil in such containers was saturated with phosphate or tris-HCl buffers (pH 7.4, 0.1 or 0.2M). After three hours the pH of the soil was measured and found to vary between 3.9 and 5.5. It was concluded that the buffering capacity of the soil was sufficiently high to warrant the use of a mild alkali for the purpose of neutralisation. Accordingly, a saturated solution of $Ca(OH)_2$ was prepared and filtered; soil columns were percolated with various volumes of alkali and left in a saturated condition for 30 minutes to 3 hours. It was found that 25 ml of the alkali brought the soil pH to 7.6 in all samples in 30 minutes. This treatment was adopted as the standard growth-promoting stimulus and was applied simultaneously to all samples by means of the pedostat described later (p. 219-225).

E (2) Measurements of activity

Problems in the choice of a suitable growth-related parameter in a study of this kind have already been elaborated (p. 79). The importance of catabolic activity has also been stressed (p. 111). Since the enzymatic method of demonstrating catabolic activity was inappropriate to this soil (p. 127 an alternative means of accomplishing a similar end was sought. Techniques which depend on enumeration are necessarily destructive and if they are used it is necessary to set up a series of parallel soil cultures so that changes in cell numbers may be assessed over a period of time. While such methods are valuable, they are open to the problem of temporal heterogeneity in the samples. Thus although activity may be initiated simultaneously in all samples, and though all samples may follow essentially similar growth kinetics, it is possible that slight variations in growth rates and response times may result in between-sample heterogeneity. This would present problems in the interpretation of a composite growth curve made up from the parallel cultures sampled at different times. At early stages in such experiments, a reasonable degree of synochrony may be expected and such problems are not likely to be great. As time goes on however, growth curves in replicate samples may be expected to diverge as a result of small physical and chemical differences between the samples. Thus, some means of monitoring activity continuously without sample destruction would be advantageous. This could give information on the degree of synchrony between replicate cultures and would assist in the interpretation of growth curves based on enumeration techniques.

Methods of continuous activity monitoring which have been applied in various branches of soil microbiology are based on some of the principles of respirometry. Exchange of oxygen and carbon dioxide with the atmosphere represents a catabolic activity parameter which may be simply measured with a minimum of disturbance to an intact sample. In this study only CO_2 evolution was measured since correlation of O_2 uptake with other activity parameters is difficult when a complex microflora possessing a variety of energy-yielding pathways is considered. The oxygen-uptake of such a system will also largely depend on the aeration status. However, under most conditions in soil it may be assumed that growth will be accompanied by CO_2 evolution. Provided the conditions are kept as constant as possible, it may be feasible to derive empirical correlations between CO_2 production and enumerative data. In conditions where only a small proportion of the organisms were respiring, such a correlation with oxygen uptake would be more difficult.

Method

The collection and analysis of CO_2 are described in Appendix B.

Results

Triplicate soil columns and controls were set up as described. A number of parallel identical soil columns were set up for purposes which will be described later (p.142 and 152). The soil was neutralised and held at 22 ± 2°C. Thereafter, CO_2 produced in every 12 hour period was determined titrimetrically. The results were calculated as nanomoles CO_2/g dry soil/hour and the mean for the three columns is plotted against median time in Figure 16.

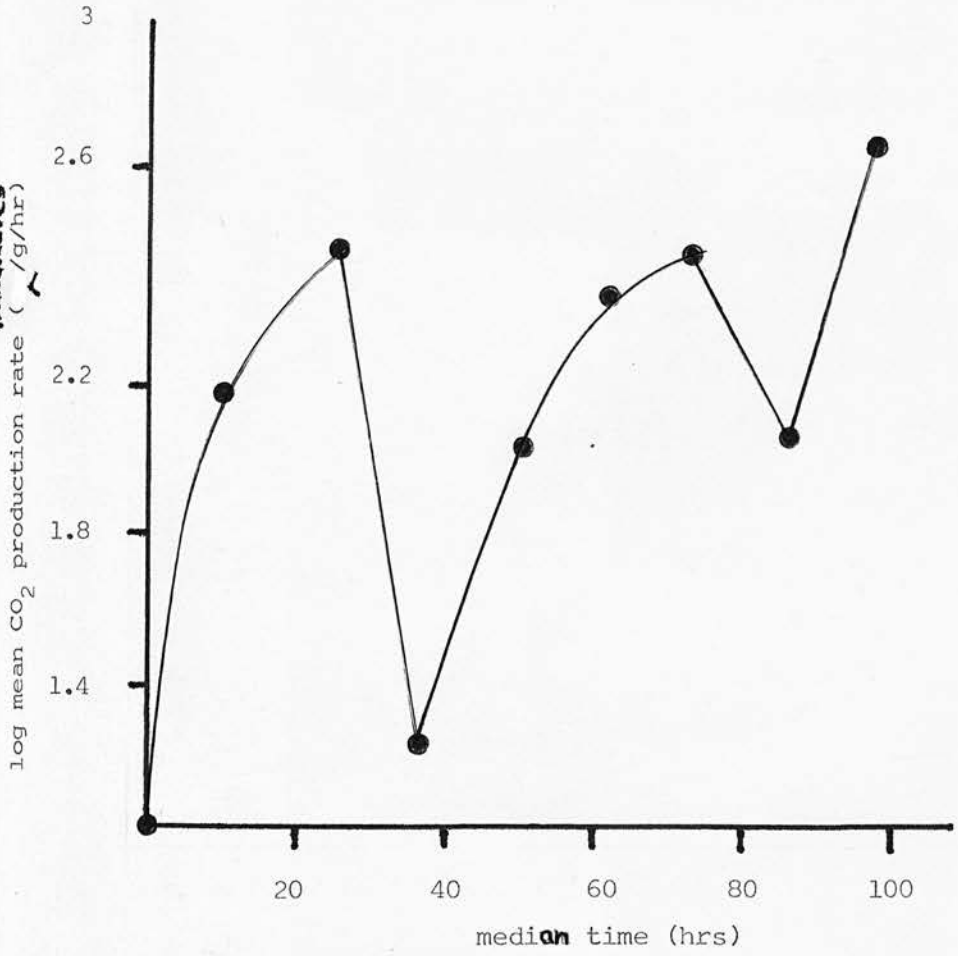


Figure 16. Response in terms of CO_2 production rate of a model soil microflora to a stimulus at one point in time.

The scale of variation with time was in terms of tenfold increases and decreases; accordingly, the rate of CO_2 production was plotted on a $\log_{10}$ scale.

Discussion

It can be seen from Figure 16 that activity during the experimental period took the form of at least two clearly defined bursts which could correspond roughly to sigmoid biomass increases if the relationship

$$bd \propto [CO_2] \qquad (19)$$

is assumed where bd is the biomass of actively dividing cells. This assumption is a crude approximation, but in the present circumstances was thought probably to be valid as a first approximation. It explained simply why CO_2 production rate dropped precipitously from maxima to minima rather than forming a plateau which might have been expected if CO_2 production were directly related to the total number of living cells present. If (19) is accepted, then it follows that $dbd/dt = kd[CO_2]/dt$ and Figure 16 illustrates the kinetics of the mean rate of biomass increase on an arbitrary scale. It would appear therefore that sequential community activity takes place in the model soil stimulated at one point in time. (This observation was repeated on a number of occasions.). This pattern of CO_2 production could be explained by either a stepped growth curve type of biomass increase incorporating a number of sequential sigmoid increases, or an oscillatory change in biomass with no nett increase outside the amplitude of the oscillation. From Figure 16., the period of such an oscillation would be expected to be ca. 40 hours.

E (3) The estimation of microbial biomass in the model soil subjected to a point stimulus

The difficulty of knowing which parameter of microbial community total size (i.e. weight or number of cells) should be estimated (p.54) necessitates an investigation of both parameters and their inter-relationship. Accordingly, three soil samples were investigated. These samples had received the same treatment at the same time as the samples shown in Figure 16. The three were chosen from different points on the activity curve; cell volume frequency estimations and the contributions of groups of micro-organisms of different size to total cell numbers and total cell weight were made. These allowed estimations of mean cell volumes and their variation to be made.

Method

(1) Cell counts and measurements

A 'Vickers' binocular microscope was used with a micrometer scale inserted in one eyepiece and a gelatin disc with a scored square in the other eyepiece. The sides of the square were measured in terms of eyepiece micrometer scale units (e.u.); the scale itself was calibrated against a stage micrometer with a graduated millimetre scale. The eyepiece scale was such that 1 e.u. $\equiv$ 0.625 µm subtended on the slide. The delimited area in the other ocular subtended an area of 73^2 eu^2 on the slide.

It was necessary to choose size intervals into which counted organisms could be classified. Those chosen, and the corresponding cell volumes are indicated on p.144. The choice of size intervals was arbitrary and based on a preliminary examination of the slides.

(2) Sample preparation and total count computation

Soil samples were prepared and incubated as described on p. 138 For the preparation of Jones and Mollison films the whole contents of a single soil column were washed into 10 ml calgon; homogenisation was as described on p. 87. 1 ml of the ultimate suspension was mixed with 2.5 ml agar (p. 94) prior to moulding. If 'x' is the dry weight of soil in the column, 'y' ml the preparation volume below one field of view , c = mean count/g soil was obtained from 'm', the mean count/field by the relationship

$$c = 17.5\ m/xy$$

A total > 300 organisms were counted and measured on three replicate films for each sample.

Results

Soil column (3) was sampled at 0 hours (Figure 16), (16) at 3 hours and (27) at 44 hours. The mean cell volumes obtained were (3) 4.85 μm^3, (16) 5.06 μm^3 and (27) 3.57 μm^3 giving an overall mean of 4.49 μm^3. The experimental data for column (3) are shown below; the results for (16) and (27) were essentially similar.

Table 6. Cell volume frequency data (column (3) - see text)

Size category (e.u.)	volume (μm^3)	median volume	Mean count/field	count x 10^{-7}/g soil	vol x 10^{-7} μm^3/g soil (median)	numerical % representation	% contribution to total volume
cocci.(dia.)							
1 - 2	1.02-8.18	4.6	20.2	211.6	973.4	70.2	66.5
2 - 3	8.18-27.6	17.9	1.4	14.66	262.4	4.86	17.9
3 - 4	27.6-65.5	46.6	.03	0.31	14.5	0.1	0.99
4 - 5	65.5-128	96.8	.05	0.52	50.3	0.17	3.44
rods (l x d)							
1 - 2 x $\frac{1}{2}$	0.32-0.512	0.42	4.08	42.73	17.9	14.18	1.22
2 - 3 x $\frac{1}{2}$	0.512-0.704	0.61	0.0	0.0	0.0	0	0
3 x $\frac{1}{2}$-2 x 1	0.704-2.56	1.63	1.82	19.06	31.1	6.32	2.13
2 - 3 x 1	2.56-3.32	2.94	0.28	2.93	8.6	0.97	0.59
3 x 1-3 x 2	3.32-17.4	10.4	0.87	9.11	94.7	3.02	6.47
3 - 4 x 2	17.4-20.5	19.0	0.05	0.52	9.9	0.17	0.68
			Totals:-	301.44	1463	100%	100%

Variation around the mean cell volume figure between the three sample means amounted to ca. 15%. However when the volume ranges from which the sample means were calculated were considered, this constituted a range of ± 45, 66 and 69% around the sample means. Taking a mean range ca. ± 60% gives some indication of the number/volume conversion factor error. In such terms, no significant change was detectable in the mean cell volume throughout the activity cycles in Figure 16.

Figure 17 a-f indicates the disparity between the contributions to total numbers and total volumes (hence weights) of the various size intervals of micro-organisms. As mentioned above, biomass estimates made by the method described were too insensitive to differentiate quantitatively between changes in numbers and changes in biomass. However, the importance of the disparity cannot be overlooked since other methods of estimating activity - or biomass - correlated parameters will be biased more or less depending on which community parameter is preferentially selected by the method of analysis. Thus in enumerative procedures involving dilution stages, it is the most numerous component which is preferentially selected; in activity measurements made by the analysis of CO_2 evolution, it is the component which produces the most CO_2 which is preferentially selected.

Discussion

(1) Counting and measurement of cells

The usual maxim of a total >300 organisms per film (Jones and Mollison, 1948) was adopted for routine counting. This necessitated the examination of 10-30 fields at the dilution used.

Examination was restricted to this number simply because of the time necessary to carry out the counts and measurements. This procedure obviously did not give satisfactory sampling of the minority components (judged numerically). For example an organism occurring at a frequency of 0.5×10^{7}/g would necessitate the examination of 6,000 fields to show 300 cells. (It is interesting to note that such an organism would be quite capable of appearing numerically dominant by plate-count methods.). A lower dilution would not be practicable since numerically minor components would be masked by more numerous organisms. This line of reasoning leads naturally to consideration of the limits of detection of the method. If ca. 20 fields/film is considered a reasonable number to examine, ca. 1.6×10^{9}/g is the order of count to be expected if 300 organisms are counted at the dilution used. Thus, only the whole community is adequately sampled; i.e. those components which make up <100% of cell numbers cannot be adequately sampled and proportions, counts etc. mentioned in the results section above must be regarded as crude approximations. Taking no account of the statistical distribution of organisms per field (p. 84)any organism occurring at a frequency of <1/20 fields will not be detected at all; i.e. 0.5×10^{-7}/g is the theoretical lower limit of detection and soils containing less organisms than this would give no count at the dilution used. The effect of the shape of the frequency distribution of organisms in fields on these conclusions is not to alter them, but to make them more uncertain.

Accepting the fact that the method is highly selective with respect to numerically predominant organisms, some consideration

must be given to difficulties experienced in cell measurement. As can be seen from Table 6, little difficulty was experienced in allocating the upper volume interval of classification. This is because, as cell size increased frequency decreased rapidly. The lower volume interval of classification was however much more difficult to ascertain since frequency increased even as size decreased below the limits of optical resolution. It was arbitrarily concluded that the smallest objects experienced were not bacteria and were probably fragmented or partially decomposed micro-organisms. As such, they would be better considered as part of the soil organic matter than as part of the microflora. Hence it was clear that some lower size limit must be arbitrarily set below which bacterium-like objects would be ignored. This was set at cocci <1 e.u. in diameter (1 e.u. $\equiv$ 0.625 μm); this was the smallest size which could be measured satisfactorily with the eyepiece scale. This may be taken as an optical limit to the accuracy of the total count. Recent electron-microscope studies (Mishustina, 1973) on marine muds have revealed a variety of sub-optical micro-organism-like objects. The real status of such objects however remains equivocal.

During the differential counting procedures, sub-totals of organisms belonging to each pre-ordained size interval were entered in a prepared table. This involved accumulating the total number of organisms in each class-interval on a manual tally-counter. There was a possibility of double allocation in this procedure, especially with the smaller organisms. The relatively wide intervals employed were the best compromise

between precision and tedium in the circumstances. However, if organisms could not be simply classified, there was a tendency to ignore them since they successively failed to be included in class intervals and thus tended to be included with those organisms already counted. To this extent the measurements were subjective and care had to be taken to avoid lack of reproducibility in this respect.

(2) The relationship between volume and numbers

A weighted mean cell volume was calculated by the standard procedure:

$$([f_1x_1 + f_2x_2 \ldots\ldots\ldots + f_nx_n]/[f_1+f_2 \ldots\ldots\ldots\ldots + f_n])$$

$f_{1,n}$ being the frequency and $x_{1,n}$ the midpoint of the appropriate class interval. As indicated earlier, when the class interval width was taken into consideration the mean cell volume of 4.49 μm^3 did not change significantly throughout the activity cycle. This is not to say that the community did not change qualitatively as well as quantitatively. At any instant of time, the metabolic activity manifested by the community will be the sum of the products $\not\phi$ (biomass x growth rate) of each active population component. ($\not\phi$ signifies an unknown function related to carbon assimilation efficiency). From Figure 18 b, d and f it can been seen that the community appears to have changed qualitatively (as would be expected) during the course of the activity cycle.

Since the mean volume as calculated above will be weighted most by the highest value of 'f', the numerically dominant component

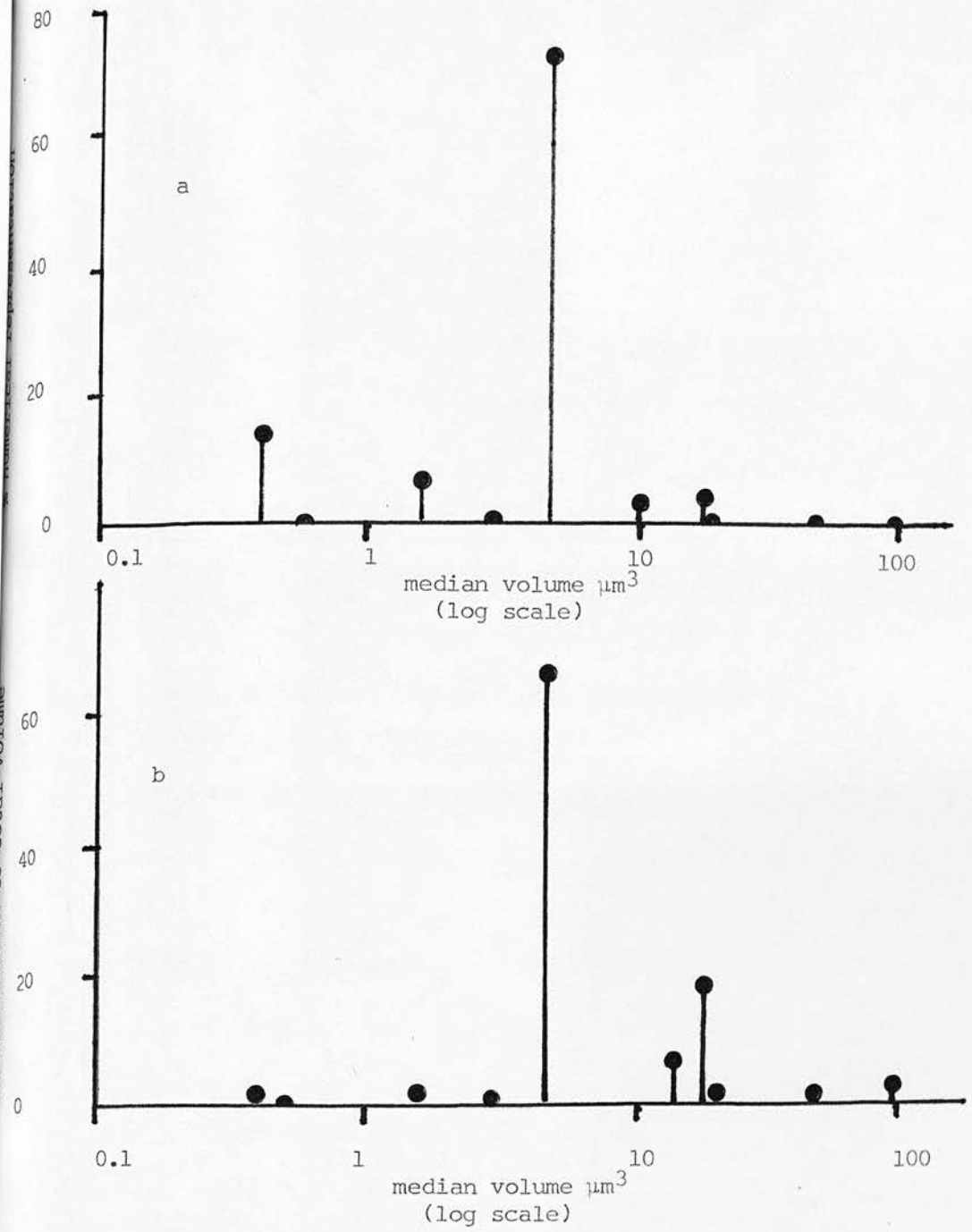


Figure 17. Proportional representation in total numbers and total volume of various cell volume categories.

a,b sample 3
c,d sample 16
e,f sample 27

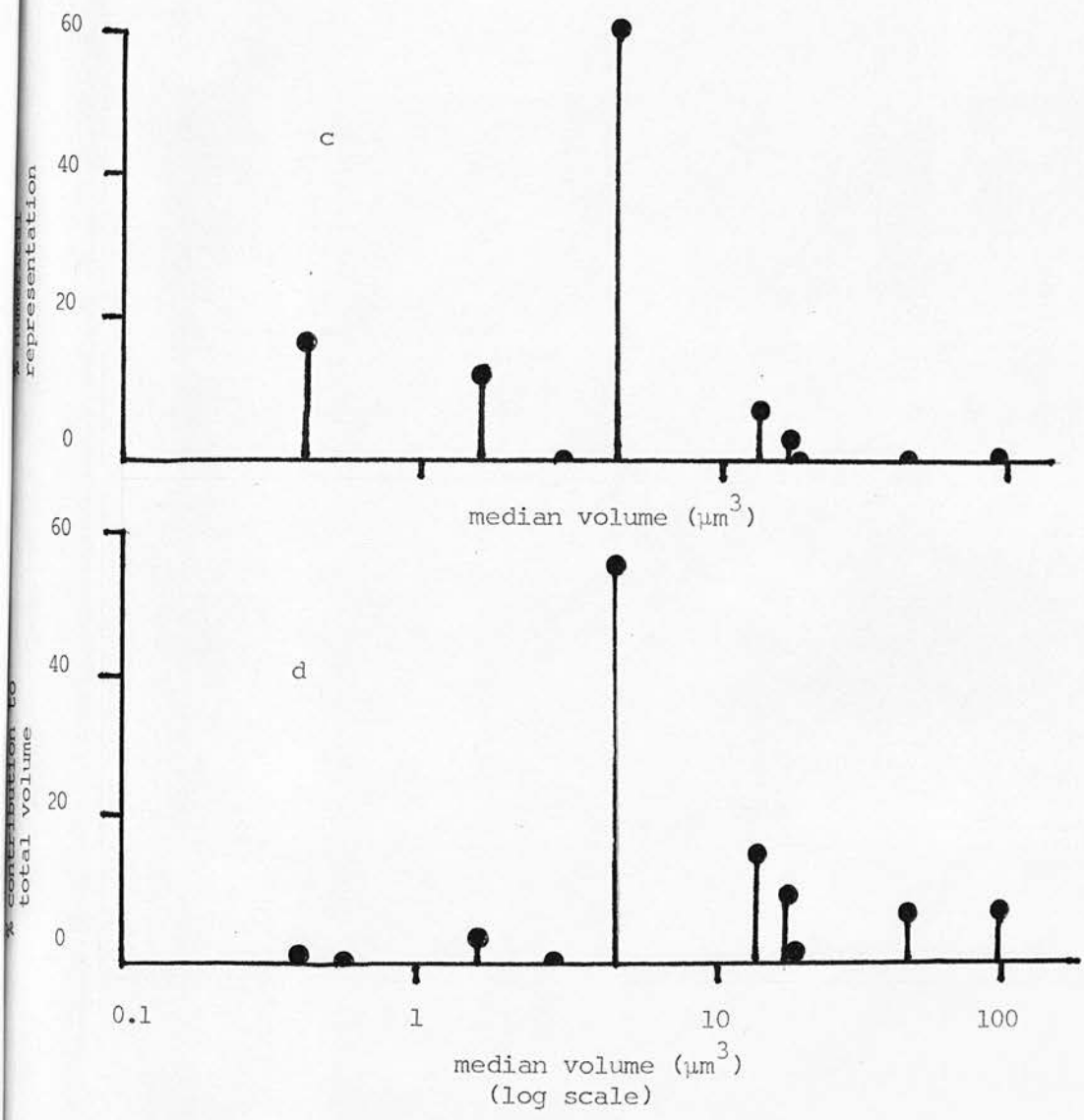

60
40
20
0
% numerical representation
c
median volume (μm3)
60
40
20
0
% contribution to total volume
d
0.1
1
10
100
median volume (μm3)
(log scale)

Figure 17.

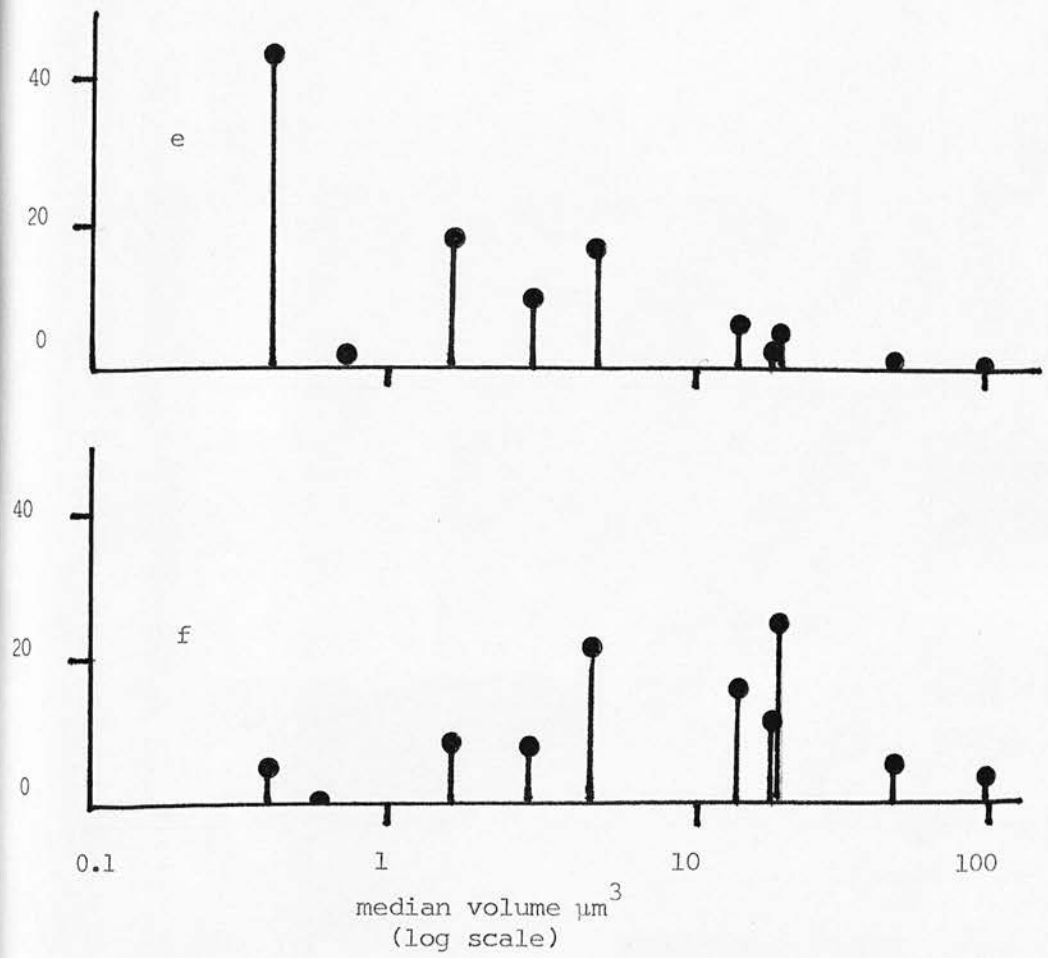


Figure 17.

would tend to equalise the mean cell volume figures for the three samples unless it changed by a large amount during the course of the activity cycle. It can be seen from Figure 17 that the tendency was for the initially numerically predominant component to be progressively superceded by components of smaller size. Thus the final mean cell volume figure is weighted towards components of smaller cell size and at 3.57 μm^3 is noticeably less than the mean for all three samples (4.49 μm^3).

(3) The community fingerprint concept: application of volume frequency data

It was concluded earlier (p. 33, 98) that qualitative characterisation of a microbial community in terms of isolated organisms is unlikely to be very useful when based on plate-count and classical taxonomic data. Accordingly, some other means of describing a community (or population) must be found which can be used to differentiate one community from another or to ascertain whether any changes are taking place in community structure. Ideally such a characterisation should be an ecological one based at the population and community level rather than at the species level. It should be stressed that there will be as many "fingerprints" as there are measurable parameters which can be divided into appropriate class intervals. Each fingerprint will be biased in favour of that component making the greatest contribution to the total community measurement; (i.e. that component will be measured with the greatest degree of certainty). This component may not be the most important one; take for example the case of a numerically dominant population

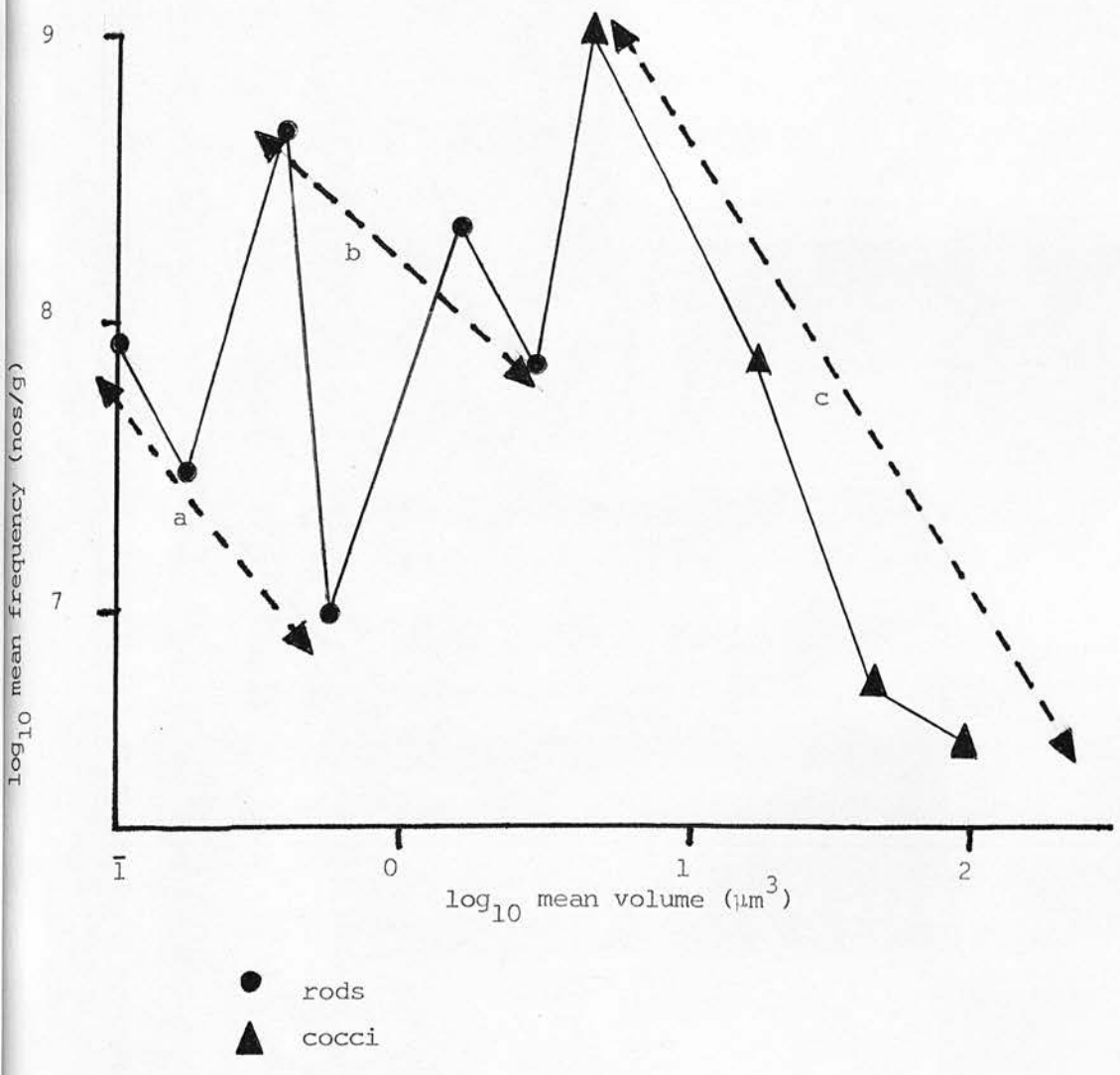


Figure 18. Average numerical volume frequency fingerprint for the model microflora.

of small organisms which carries out a small fraction of the metabolic activity of the community. It should be stressed that the dominant component with respect to one specific metabolic parameter (e.g. CO_2 production) need not be dominant with respect to another even related parameter (e.g. oxygen uptake).

Application of the ideas outlined above to the volume distribution data gave rise to Figure 18 which is an average numerical volume frequency fingerprint for the community over the experimental period. The $\log_{10}y$ scale was used because of the high inherent statistical variation in total count figures, while the $\log_{10}x$ scale compressed the wide range of volume intervals to workable limits. In reality, Figure 18 is a bar chart; however, joining consecutive points gives a picture which is qualitatively more acceptable and can be more easily assessed visually. There appear to be three clusters of volume frequency a, b and c. That these are probably partly of a physical rather than biological origin is indicated by the fact that 'c', the most coherent cluster, is made up solely of spherical organisms.

It was mentioned earlier that the weighted mean cell volume did not change significantly over the experimental period. However, the composition of the community did change detectably in terms of volume frequency (Figure 17). Community fingerprints along the lines of Figure 18 were drawn up for the three samples and are presented in Figure 19. The relative differences between the samples in terms of which components have increased and which have declined can be immediately assessed. Differences in the fingerprint between sample (3) and the other two are quite

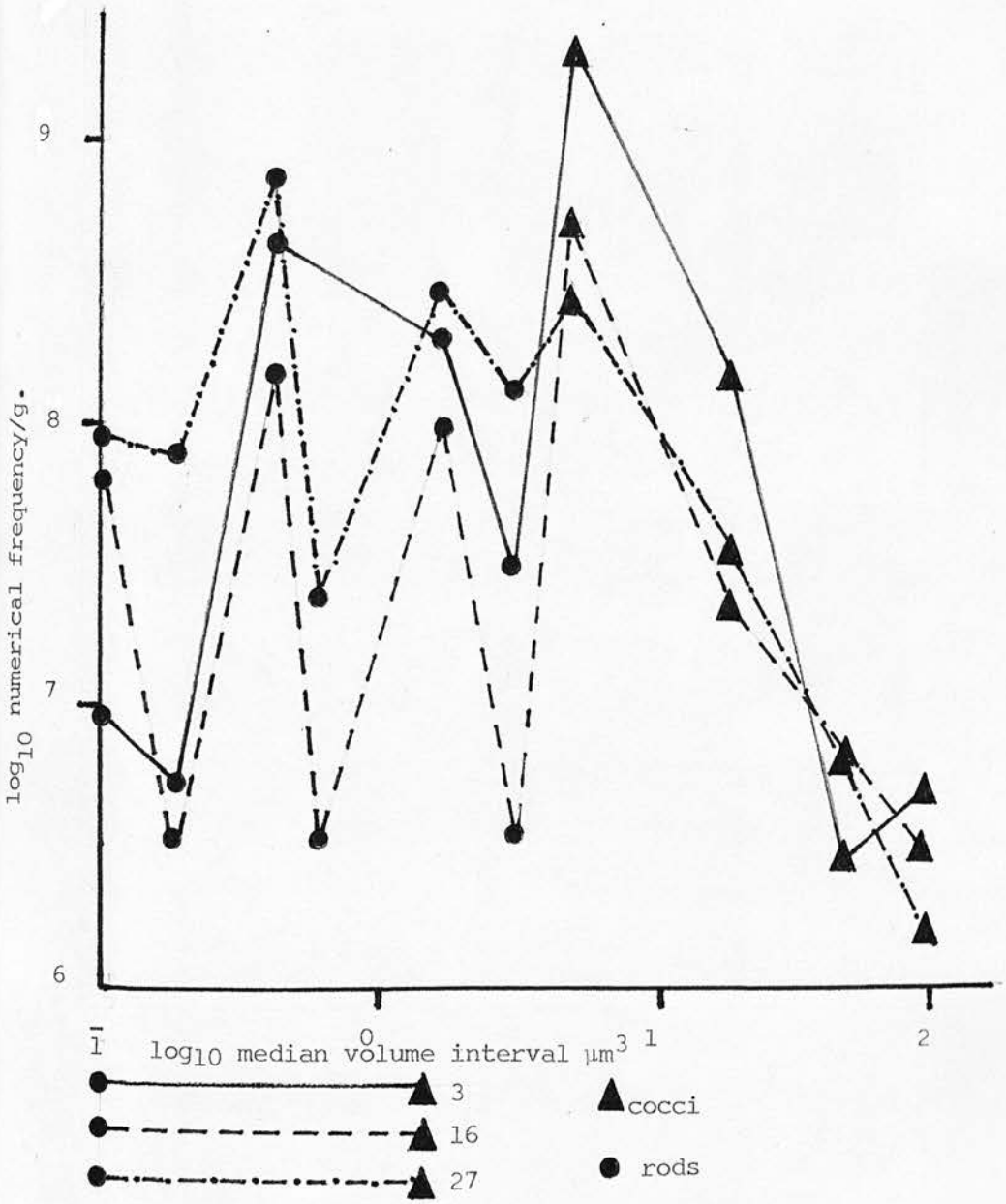


Figure 19. Community fingerprints based on numerical volume frequency.

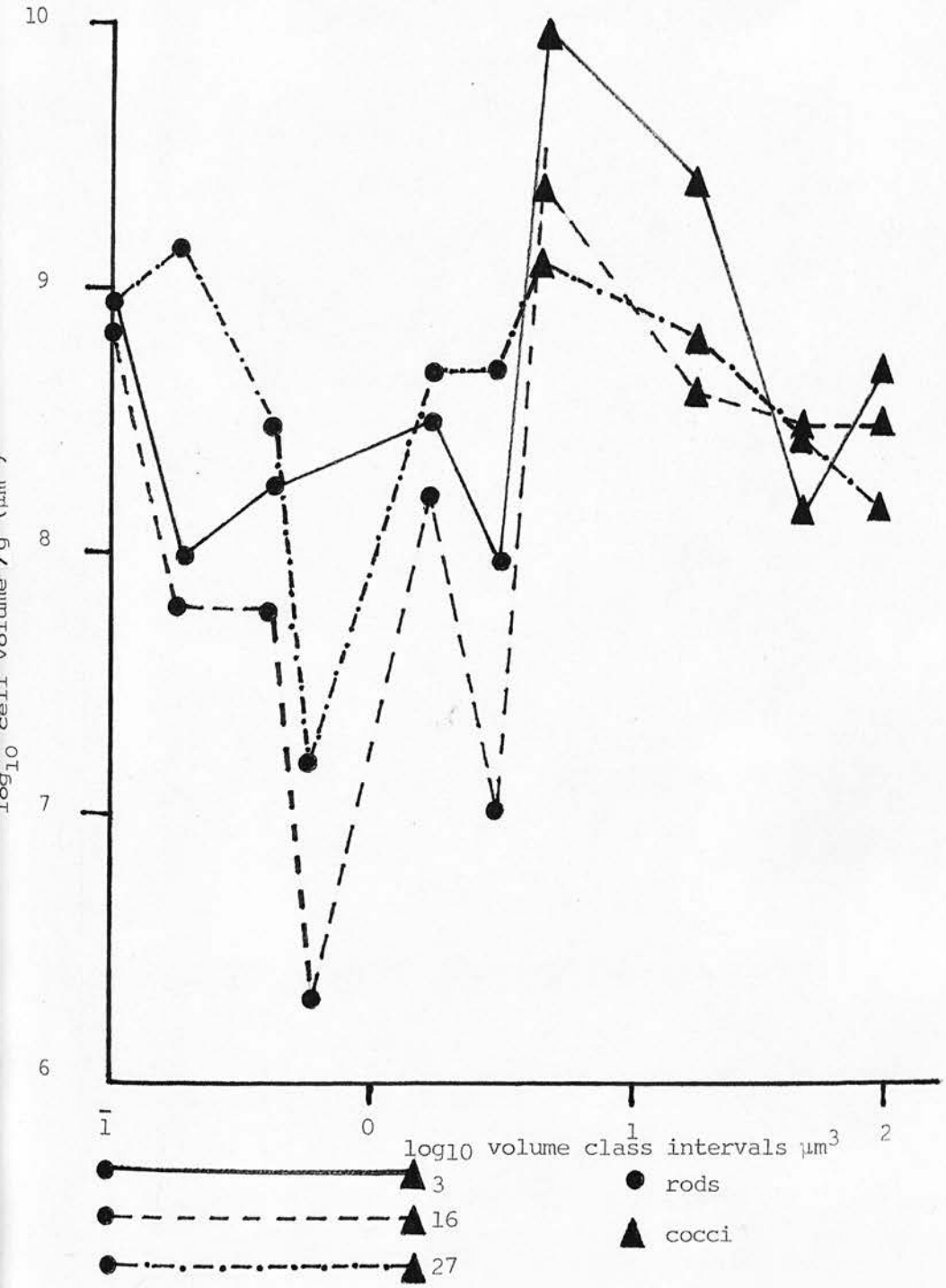


Figure 20. Community fingerprints based on volumetric volume frequency.

pronounced; (3) lacks a component present in the other two samples and exhibits a fingerprint outline quite different from those of (16) and (27) which are similar.

The criterion of differentiation between components in Figure 19 is numerical dominance. In biomass terms, Figure 19 may be replaced by Figure 20 as a volumetric (rather than numerical) volume frequency fingerprint. The differences in fingerprint are now weighted according to biomass and are more clear-cut than those of Figure 19. The main conclusion to be drawn from Figures 19 and 20 is that the initial community (3) becomes progressively more complex as the activity cycle progresses. This conclusion may also be drawn from Figure 17 b, d and f where it can be seen that more and more components gradually make up larger individual proportions of the total weights of cells present. This is interestingly different from the normally expected zymogenic response by a small number of species swamping out the effects of the rest of the microflora. It implies that some meaning can be attached to community structure and that it may be expected to be a reproducible property.

E (4) The relationship between growth and activity in the model soil subjected to a point stimulus

The analysis of the point stimulus experiment was concluded by a consideration of the biomass changes occurring during the cycles of activity noted in Figure 16. Total counts were carried out by the Jones and Mollison technique and the mean cell volume factor of 4.49 μm^3 was used in conjunction with assumed live weight microbial specific gravity of 1.0 (Clark, 1967; Parinkina, 1973) to obtain total microbial biomass estimates in a series of soil columns set up in parallel to those described in Figure 16.

Results

The curve d $[CO_2]$/dt vs time in Figure 16 was plotted using median time values i.e. the time plotted is the mid-point of the sampling interval. For comparison with biomass change, this is confusing since the median time plot tends to displace maxima and minima from their true position on the 'x' axis. In Figure 16 this is not relevant; it is simply the existence of maxima and minima which is being demonstrated. A more realistic approach in the present case is to consider a curve of cumulative CO_2 production plotted at real sampling times. Such a curve is shown in Figure 21 along with biomass variations.

Discussion

The biomass curve confirms the earlier conclusion (p.141) that at least two cycles of community growth and activity took place in the experimental period. The good fit of the CO_2 data and the poorer fit of the biomass data to the smoothed curves results from the former being the mean of continuous measurements on three samples and the latter being based on standing crop

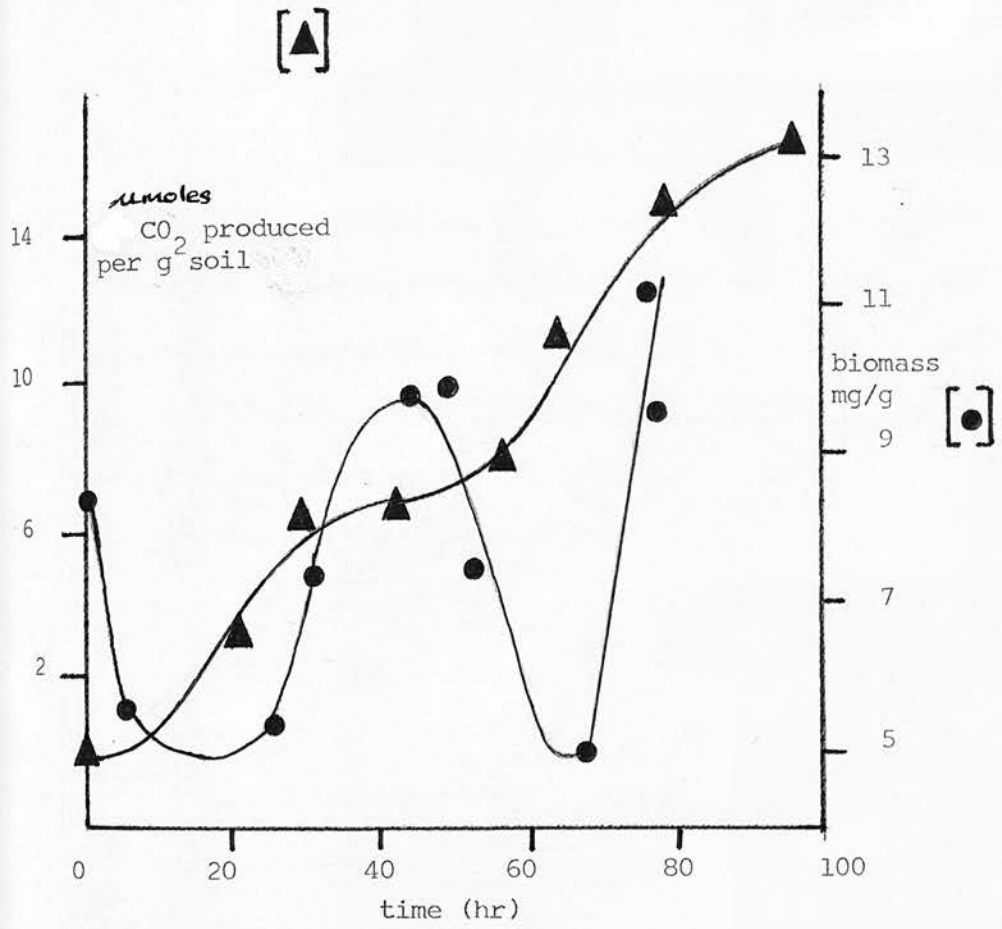


Figure 21. Variation in CO_2 evolved from and bacterial biomass contained in soil stimulated at a single point in time.

estimates from 11 single parallel samples. The biomass analysis was not extended because it was not considered likely that a large number of replicate soil columns would remain in reasonable synchrony for long periods (p.139).

The rise in CO_2 production during the initial drop in biomass suggests that activity started immediately the stimulus was applied and indicates the proliferation of some community component(s) during the first period of overall biomass decrease. The initial minimum in the biomass curve was interpreted as utilisation of dead (or inactive) bacterial tissue by living cells as a first reaction to the changed conditions. This is not surprising; the favourable nutrient status of microbial tissue has already been noted (p.21). However, even under these nutritive conditions substrate conversion efficiency is such that the first ascending portion of the biomass curve must be a result of utilisation of substrates other than microbial tissue. If, by similar reasoning, the second minimum in the biomass curve is interpreted as removal (i.e. biological consumption) of micro-organisms, a resumption of the previous lytic behaviour may be postulated although it may be that some of the first predators have become the second prey.

The phase difference of the CO_2 and biomass curves is probably the combined result of two effects: (1) the different nature of the samples used, and (2) the fact that the two functions started to increase at different times as a result of their differing sensitivities to amelioration of the environment. It has already been suggested that biomass production took place

during the first minimum. Because of the non-specificity of the total count procedure, if this was bacterial it would not be detected because of the overall drop in numbers; if it was protozoal, the method is inappropriate for its detection. The subtraction of the CO_2 component corresponding to this theoretical biomass component from the cumulative CO_2 curve results in biomass and CO_2 curves which are more correctly correlated than those in Figure 21. The time chosen for the estimation of this CO_2 component was 15 hours (Figure 21) since this was the time about which biomass increase became detectable. Because of the problems of non-synchrony mentioned above, only the period from 15 hours to the first biomass and CO_2 maximum was considered in investigating the correlation between these two parameters.

A plot of "corrected" CO_2 production (as described above) and cumulative biomass production is shown in Figure 22 where production in both parameters is adjusted to zero at t = 15 hours. It is immediately obvious that the first approximation of a linear relationship between biomass and CO_2 (p.141) does not appear to be valid. It can be seen that there is a relatively constant CO_2 production per unit biomass production (mean 5.3 μmoles/mg) up to ca. 30 hours after which CO_2 production drops rapidly towards zero while biomass continues to increase. This is unexpected if the organisms responsible for the biomass increase are responsible for the CO_2 increase. It is unlikely that these organisms were entering a retardation phase accompanied by reduced CO_2 production since biomass is still increasing at close to its maximum rate when cumulative CO_2 production is reaching a plateau (i.e. absolute production is approaching zero). Alteration of the

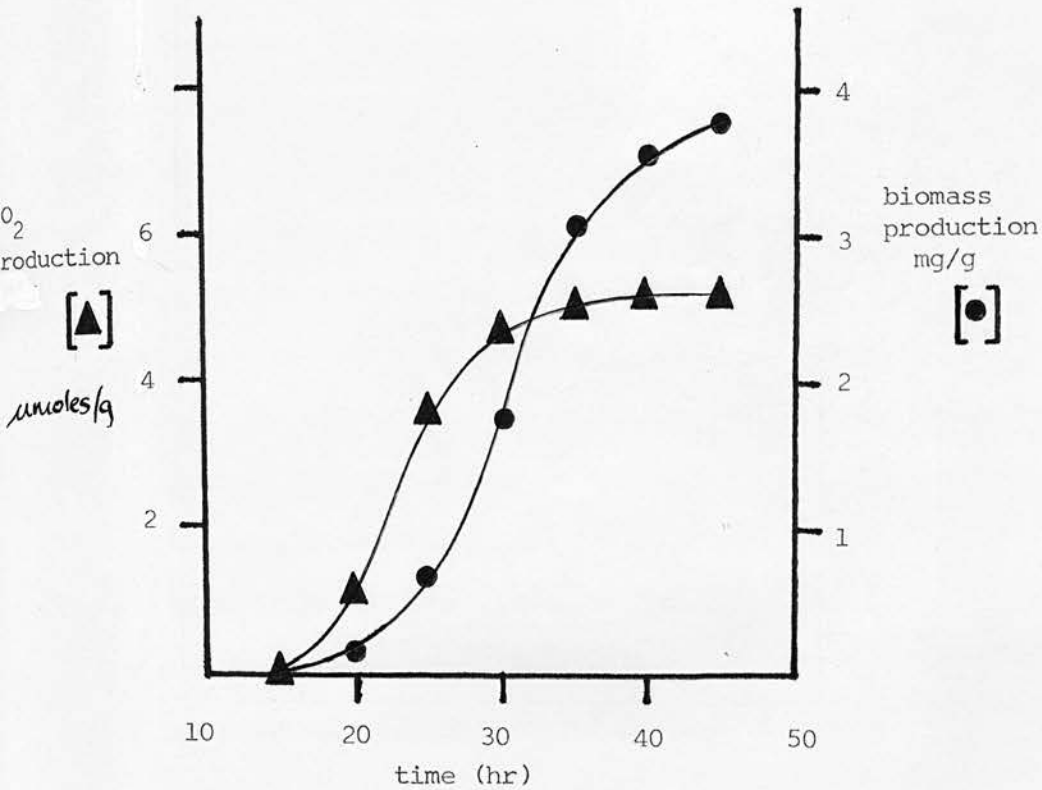


Figure 22. 'Corrected' CO_2 production and cumulative biomass production from 15 hr (Fig. 16).

environment by metabolites may be expected to alter the metabolic pattern in a versatile population; however in the case of a mixed microflora growing on varied substrates it is difficult to predict whether this would be accompanied by increased or reduced CO_2 output. The most attractive interpretation is that the component of the community responsible for the detected biomass increase is not the component responsible for maximal CO_2 production. A plausible alternative explanation is that the continued biomass increase and drop in CO_2 production is spurious and a result of between-sample heterogeneity. It was concluded that the linearising assumption of correlation between CO_2 and biomass was an oversimplification; this does not rule out the possibility that in specific components of the community, CO_2 and biomass production may be approximately directly related. Since one cycle of CO_2 production does appear to be accompanied by one cycle of biomass production it seems that there is some (undefined) correlation between the two parameters.

In terms of community function, it was earlier concluded that catabolic activity was likely to be of prime importance (p. 112). In terms of maintainence of community structure (homeostasis), differential biomass changes are probably of prime importance. Thus the dilemma of which parameter to measure in a study of community dynamics has been accentuated rather than resolved by the point-stimulus experiment.

Some limited information on the dynamics of biomass changes soon after a growth-promoting stimulus can be gained from Figures 21 and 22. Thus during the period of detectable biomass

increase, a mean tissue production rate of 0.125 mg/g/hr was noted with a maximal rate of 0.24 mg/g/hr. Periods of increase appeared on the basis of available evidence to alternate with periods of decline, probably resulting in the long term in a net zero real production rate commensurate with a positive cumulative production rate. In the experimental period, standing crop estimates varied from ca. 2.75-7.25 mg/g. In pure-culture terms, the mean and maximal biomass production rates correspond to average generation times of 20.1 and 13.8 hours respectively. If these figures are to be regarded as normal in soil, the significance of Postgate's (1973) report that the viability of chemostat-cultured Klebsiella aerogenes was greatly reduced at very low dilution rates must be carefully assessed. The maximal biomass production rate quoted above is roughly equivalent to a dilution rate of .03-.05 $hour^{-1}$. If Postgate's graphs for K.aerogenes were directly applicable to the soil bacteria studied, viability would vary from 50-100% depending on the limiting nutrient. This assumes that the cells in soil are growing under constant nutrient limitation. Here the divergence of the soil from a chemostat is of importance. The possibility that growth was limited by predation has been indicated; if alternatively growth stopped as a result of nutrient limitation, the limiting situation is on the shoulder of the biomass curve. In other words, the viability of the organisms when growing at their maximal reproductive rate is not likely to be reduced simply because they are growing slowly compared to laboratory pure cultures.

An alternative approach to the analysis of the point-stimulus experiment is to make use of the conclusions of Payne (1970), Bell (1972) and Abbott (1973) on the calculation of microbial tissue yields. The amounts of biomass produced from a given amount of an organic compound can be predicted from its carbon or available electron content (Abbott, 1973). According to Bell (1972) a constant yield factor Y_c = 1.1 g biomass/g carbon transformed was applicable to growth on sugars, polyhydric alcohols and paraffins. The constancy of Y_c during growth on such substrates is due to a constant ratio of carbon assimilation to CO_2 formation; according to Payne (1970) a 60% carbon assimilation efficiency was applicable to a wide variety of micro-organisms and substrates. Such conclusions have been based wholly on pure culture studies but it is interesting to note that McGill et al (1973) in a study already referred to (p. 61) found an identical conversion efficiency during the growth of a natural microflora in a field soil.

Exceptions to the constancy of Y_c may be noted during growth on carboxylic acids (Bell, 1972) where an inhibitory effect appears to reduce Y_c to 0.8. It has also been shown (Abbott, 1973) that Y_c = 1.1 cannot be applied to highly oxidised substrates such as citrate and pyruvate since the efficiency of transfer of substrate energy to protoplasm energy would have to approach or exceed 100% in such cases. Provided such restrictions are borne in mind, some crude extrapolations from CO_2 production data to biomass production and substrate turnover are possible. This of course involves the familiar linearising assumption (p. 141) of unproved validity and restricts biomass

predictions to the community components responsible for maximal CO_2 production:

CO_2 produced 15–45 hr (Figure 23) = 5.2 μmoles/g

= 5.2 x 12 x 10^{-6} g carbon/g soil ≡ 40% substrate C

∴ biomass carbon = 93.6 x 10^{-6} g C/g soil

On a dry weight basis of 53% C (Humphrey, 1968) and on the assumption that living tissue is 80% water,

$$\text{live weight production} = \frac{93.6}{53 \times 20} \times 10^{-2}\text{g} = 0.88 \text{ mg/g}$$

This is ca. 24% of the biomass detected by the Jones and Mollison method, which tends to add weight to the conclusion that the component responsible for maximal CO_2 production is a minority biomass component. It also indicates that ca. 76% of the biomass was produced without any CO_2 production. This would be an unlikely occurrence and indicates that a greater partition of CO_2 production between biomass components took place in the model soil than would have been expected from Payne's (1970) data. However, even considerably altered carbon conversion efficiencies would not alter the main conclusion that the major community component in terms of CO_2 production is probably a minor component in terms of biomass. Conversion efficiency studies could be carried out along lines indicated later (p.165) to clarify these points.

If there is any validity at all in the assumption of a constant 60% carbon assimilation efficiency, it should be possible to estimate the amount of substrate transformed by the CO_2-producing community component. Thus during the period 15–45 hour (Figure 22)

ca. 0.16 mg substrate carbon was transformed. It can be calculated from the data on p. 211 that there is an average 15.1 mg carbon/g of the model soil; thus it appears that there is ample substrate present to support such microbial growth. In this way a picture can be constructed of spates of primary substrate transformation (resulting in microbial proliferation) alternating with spates of biomass degradation. The limits and extent of microbial activity in the sealed microcosm of the point-stimulus experiment could be crudely predicted. This approach is described not because of any belief in the absolute validity of the figures, but to show the sort of analysis which would be possible if experimentally determined carbon assimilation efficiency data were available for this type of system.

Some of the limitations of the point-stimulus experiment can be avoided if continuously stimulated microfloras are considered. This approach requires specialised apparatus, the construction of which is described in Appendix C.

SECTION F:

Pedostat experiments on a model soil

F (1) Introduction

The soil samples described in Figure 16 in the earlier point stimulus experiment were used initially. After the last sampling period of the previous experiment, the pedostat was turned on to continuous flow with water in the reservoirs. Thus application of the growth sustaining stimulus was delayed until the initial flush of activity resulting from the growth-promoting stimulus had died down to some extent. This procedure was useful in that it gave some indication of the history of the microflora in each sample prior to continuous stimulation. The choice of flow-rate for the pedostat was largely a matter of convenience, but to have any relevance to reality it must represent an elution rate likely to be experienced by natural microbial communities. Initially an estimate of the average annual precipitation at the sampling site of 1145 mm was obtained from Scottish Soil Survey data. On the basis of Meteorological Office data, this was later found to be low, but may well be representative of local regions at lower altitude. Since no attempt was being made to mimic a specific environment, the value of 1145 mm was adopted as being of the correct order. This was equivalent to a mean flow rate of 0.0013 ml/min on the exposed area of soil in the columns. The machine was set to deliver at this rate; operation took the form of one two minute period of delivery per hour.

Soil incubation temperature in this and the point-stimulation experiment was maintained at 22 ± 2^{o}C. Temperature was recorded

continuously from a platinum resistance thermometer inserted into a single soil column. The thermometer was balanced in a simple Wheatstone bridge and the output fed through an amplifier to a potentiometric recorder. CO_2 production rate was determined as described (p213-218); sampling intervals were of the order of 24 hours and the continuous flow experiment was carried out for 320 hours.

Preliminary Results and Discussion

The mean CO_2 production rate as nanomoles CO_2/g/hr is shown in Figure 23 against median time. A log y scale was used to compress the amplitude of the oscillations. It is obvious that application of the growth-sustaining stimulus has altered the pattern of community activity (as expected, p.219). The activity of the continuously stimulated model system took the form of a series of oscillations of convergent amplitude and decaying period. The variations were not however as extreme as those experienced in the point-stimulus experiment. This was not unexpected since in conditions where a variety of nutrients is continuously eluted from the soil, it would not be anticipated that a high growth - and activity-rate would confer a great competitive advantage upon a specific group of micro-organisms. In point-stimulus conditions however the converse situation may occur. The probability that a relatively large concentration of nutrients was made available by the soil neutralisation procedure, and that much of this was leached out or displaced by continuous elution also exists. Alternatively, if the reduction in amplitude on application of the growth-sustaining stimulus is considered as an inhibitory effect, it could be interpreted as a global distribution of inhibitory

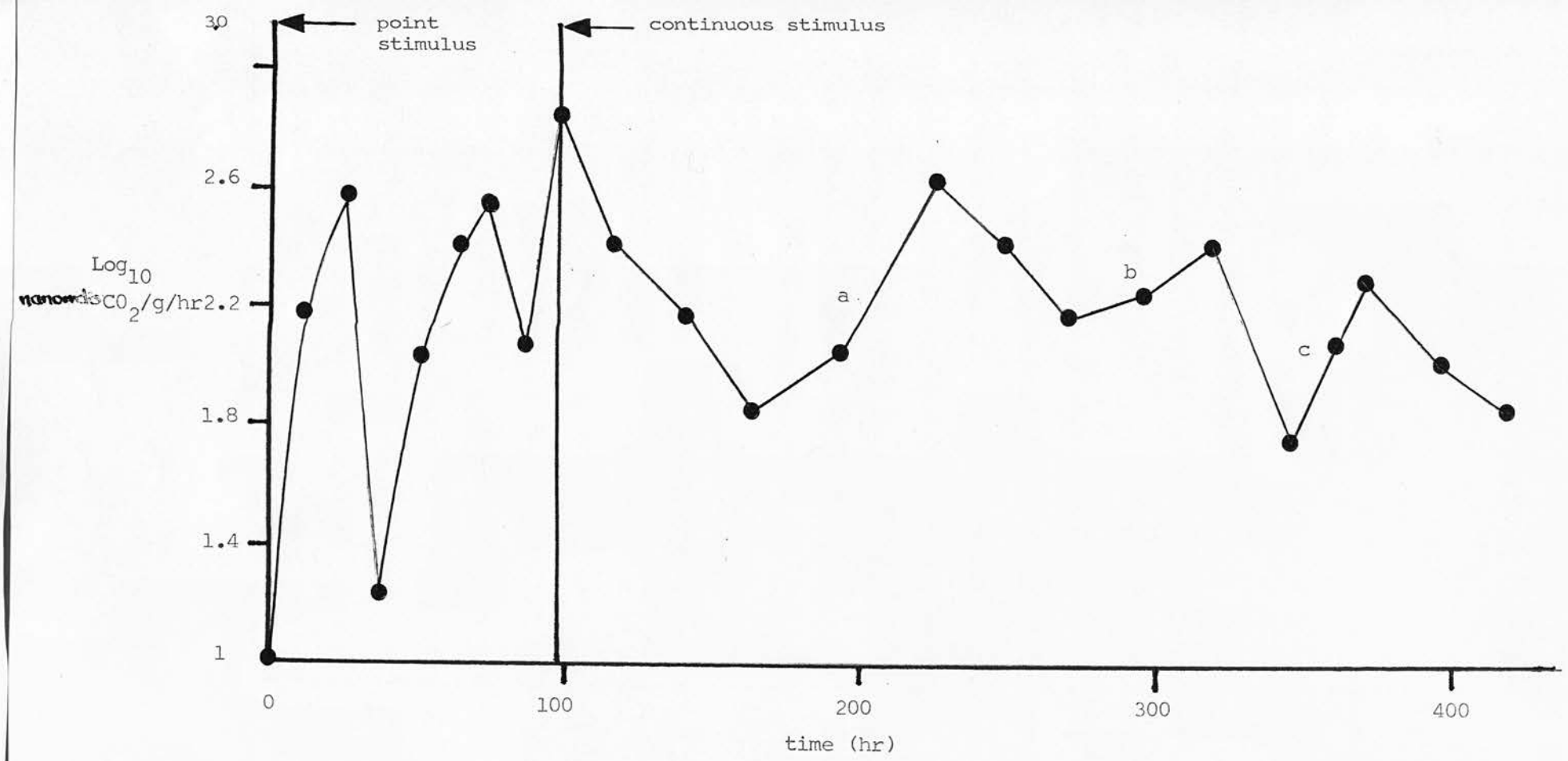


Figure 23. Behaviour of the model soil microflora subjected to continuous stimulation.

metabolites. Under point-stimulus conditions, such substances may be expected to remain close to their sites of production, their mobility being limited by diffusion rates.

The trend of the decay of the oscillations in Figure 23 is indicated in Figure 24. In the short term there are two possible courses for the continuation of this curve: (a) it may become asymptotic about some value of the period i.e. reach an equilibrium, or (b) accompanied by a progressive fall in the mean of the amplitude it may continue to drop i.e. the oscillations may become damped to extinction and the community activity eventually cease. The second possibility would be inevitable if the experiment had been continued for long enough since nutrients would eventually have become limiting in the soil. In the short term however, it is possible that some equilibrium may be reached - this would be rendered more likely by the addition of nutrients in the eluent.

If instead of mean CO_2 production rate, the mean cumulative CO_2 production is plotted against time (Figure 25), the effects of the oscillations can be smoothed out and the longer term significance of changes in activity rate be more readily assessed. It is clear from Figure 25 that after an initially rapid decline in activity rate after the start of continuous stimulation the rate appears to settle into a very gentle decrease. As in the case of the frequency, it is difficult to extrapolate this curve to a plateau (i.e. zero slope and an inactive community). It is possible however that the initially higher gradient of the curve is a result of "carry-over" from the point-stimulus while the more gently decreasing slope is characteristic of the growth-sustaining stimulus.

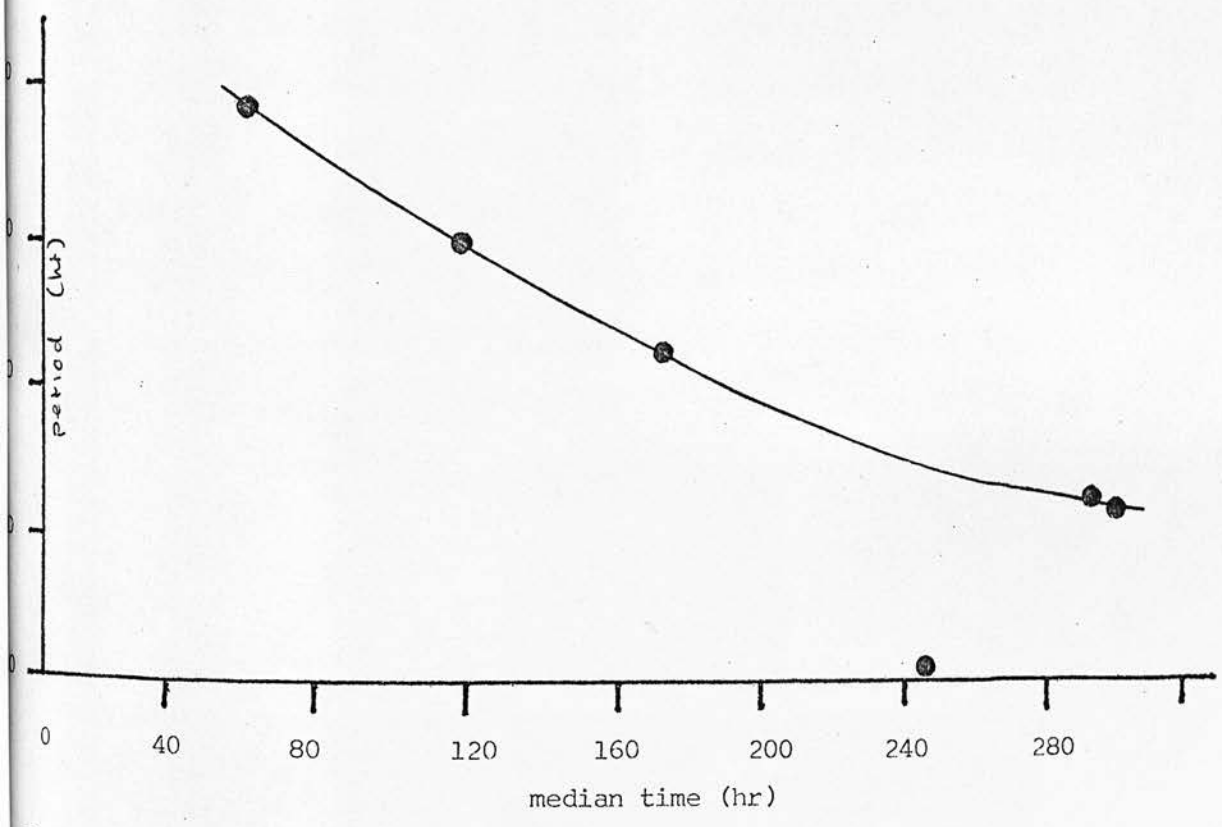


Figure 24. Decay trend of the oscillations in Fig. 23.

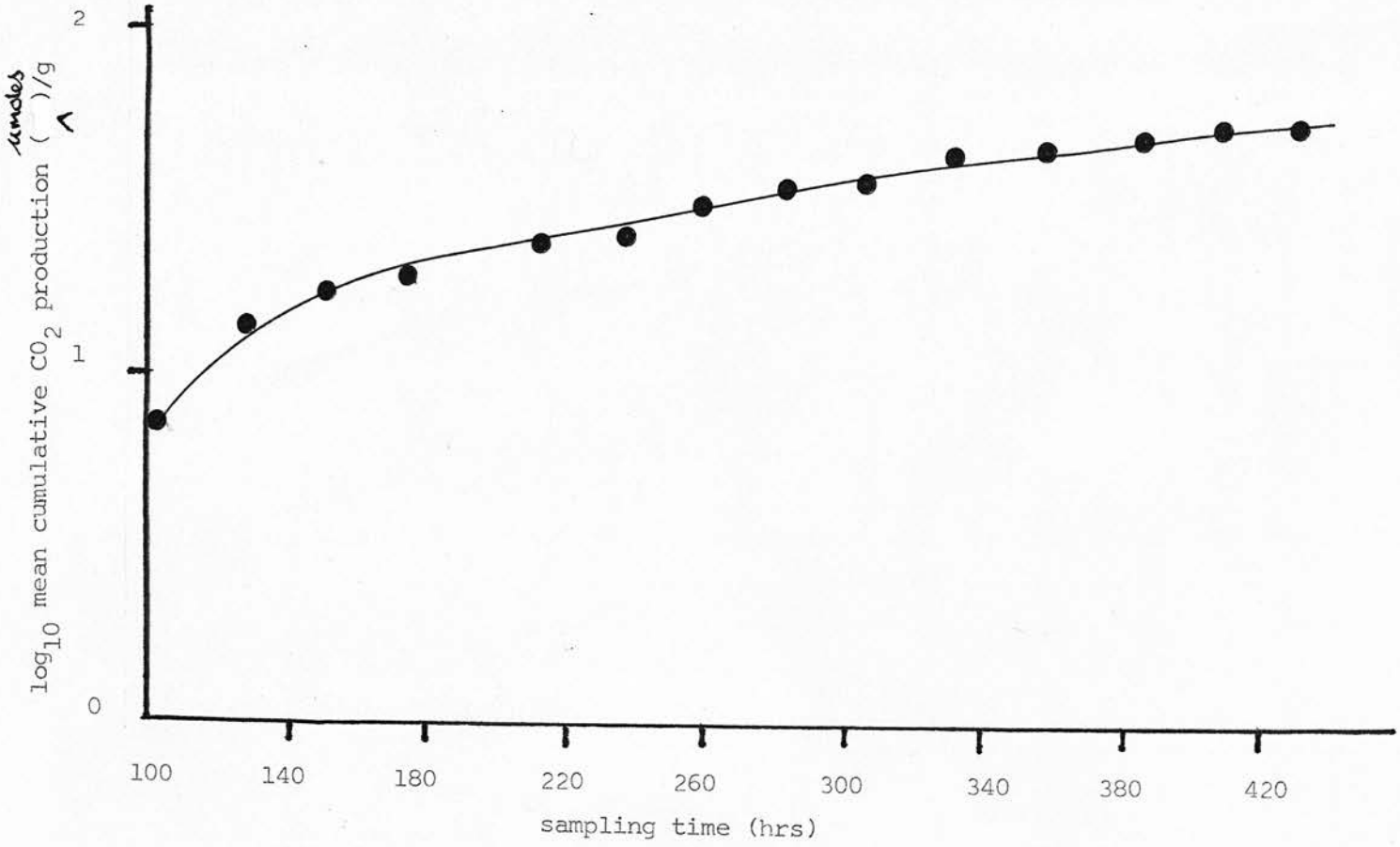


Figure 25. Mean cumulative CO_2 production from the continuously stimulated model soil microflora.

In the light of the findings of the point-stimulus experiment, it is difficult or impossible to extrapolate from activity data to biomass. Making the linearising assumption of correlation between CO_2 and biomass production (p.141) allows estimates of biomass doubling times of 41, 40 and 47 hours to be made for the ascending portions of Figure 23 on the basis of identity with CO_2 production doubling times. The validity of such figures is however impossible to ascertain, since judging by the result of the point-stimulus experiment they are referable to biomass components which since not numerically predominant do not feature significantly in the total count estimates. It is probably valid to assume from such estimates that doubling times about twice those measured directly in the point-stimulus experiment occurred in the continuously stimulated soil.

F (2) The possibility of resolving population components from community activity data

The fact that certain selected growth - or activity - correlated community parameters appear to vary periodically in a stimulated microbial community offers a tremendous opportunity for quantitative analysis of population components within the community. If the oscillations in CO_2 production rate and biomass production are considered as wave-forms, the basis of possible population resolution becomes apparent. The simplest physical analogy is the use of a prism on "white" light to resolve the various wavelengths of "coloured" light. A mathematical frequency analysis could replace the prism and community activity data could replace the white light. Such an approach dictates certain experimental conditions: (1) that the community should be at an equilibrium i.e. components oscillating at different frequencies should do so regularly and with a reasonable degree of constancy, and (2) that sampling should be carried out sufficiently often to reveal the presence of high frequency components. In its simplest form, the analysis would involve (1) deriving an equation to represent oscillations in the predominant component, (2) subtracting the derived function from the community oscillations and thus proceeding in turn to consider components which made up smaller proportions of the community activity. The process would ultimately be limited to the examination of a small number of components since experimental variations (noise) would tend to swamp minority components. In this way, a dynamic community fingerprint could be derived from activity data, the allocation of class-intervals in terms of wave-form parameters being made on the basis of experimental findings rather than convenience of

classification. Such a system would naturally be subject to the bias inherent in the characterisation of any complex system by means of a single parameter. If however, a similar analysis was carried out on the community using a second or third activity parameter (e.g. 0_2-uptake or disappearance of a specific substrate), the identification of specific population components on the basis of a known or assumed relationship between the measured parameters becomes a possibility. Consider for example the resolution of a component of frequency 'X' cycles per day from community CO_2 production rate data; if concentration of a specific (CO_2-yielding) substrate were measured simultaneously it would be expected that a component having a frequency of 'X' c.p.d. but exactly 90^o out of phase with the CO_2- derived component could be biologically the same component characterised by the two methods of analysis. (i.e. CO_2 production would be maximal when substrate disappearance was highest and its concentration was at a minimum). In such an experiment, comparison of the relative amplitudes of metabolite and substrate oscillations could yield information on the biochemical kinetics of substrate transformations in soil.

The pedostat is potentially useful for carrying out such experiments on suitably prepared model systems since substrates may be added continuously and will appear continuously in varying quantities in the effluent liquid. Provided sufficient continuously measurable activity parameters can be found, it should be possible to build up a composite picture of a stimulated model community in terms of frequency functions in various parameters. It is obvious that some population components would

appear in some such dynamic fingerprints but not in others. The fascinating possibility of gaining some insight into the interactions and functional relationships between different population components would be theoretically feasible using this approach, but in the absence of data, further philosophising is unwarranted.

F (3) The effect of pedostat flow-rate on the pattern of community activity

Three soil columns and controls were set up under conditions essentially identical to those described earlier (p. 160). The pedostat was programmed to deliver a mean 0.0023 ml/min, approximately double the flow-rate described previously. Four days after soil neutralisation the pedostat was switched to continuous flow and mean CO_2 production rates were determined over ca. 24 hourly intervals for 470 hours. The result is shown in Figure 26 (Qualitatively similar results were obtained on other occasions.). It is obvious that doubling the flow-rate has not doubled the CO_2 production although it has increased it. The most obvious difference between Figure 26 and Figure 23 is the difference in amplitude, the former showing relatively shallow cycles of variation in CO_2 production rate. It would appear from these results that growth is not primarily nutrient-limited since proliferation and activity would be expected to be more extensive in Figure 26 if this were the case. Such a conclusion points to the possibility that some homeostatic mechanism within the community may limit proliferation i.e. growth may be limited by autoinhibition. Under the higher flow-rate regime of this experiment, it may be expected that the concentration and nature of substrates in the soil solution will be different from that in the earlier experiment. It is possible that an increased variety of substrates, or lower inhibitory metabolite concentration has allowed a greater number of community components to proliferate than before. The combined effect of a large number of randomly fluctuating components need not be greatly different from the shallow oscillations shown in Figure 26 since low

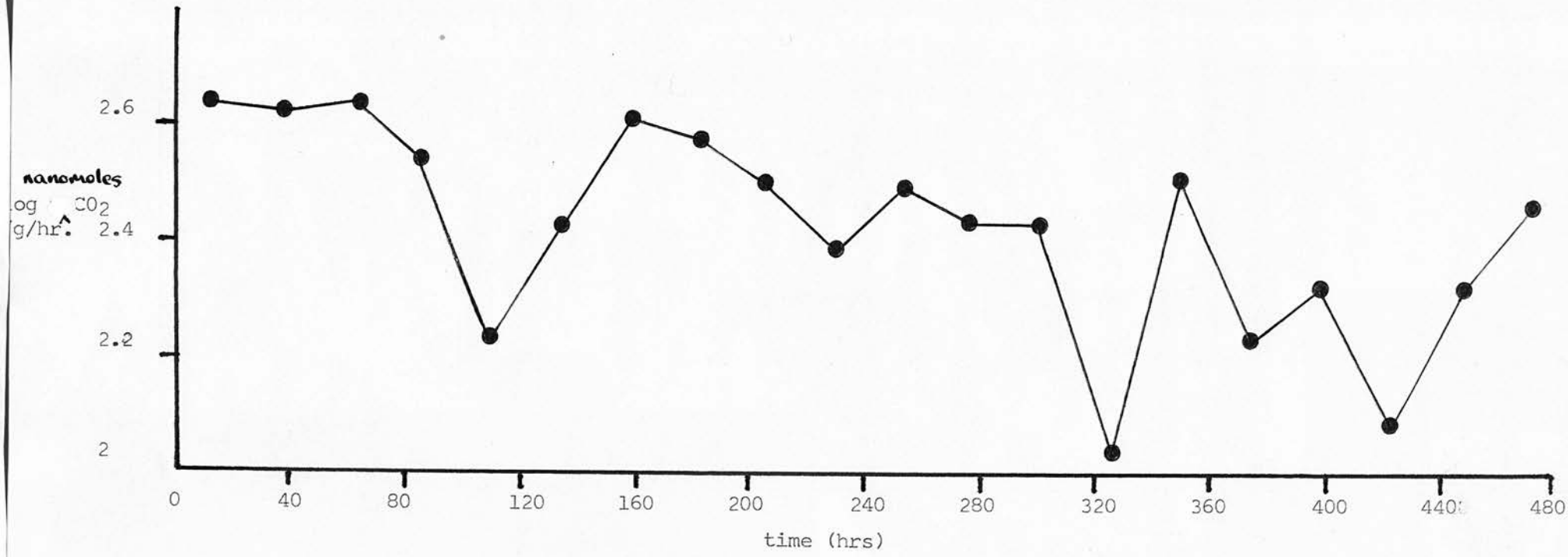


Figure 26. Behaviour of the model soil microflora when stimulated continuously at 0.0023 ml/min elution.

amplitude individual oscillations would tend to be swamped by quantitatively similar oscillations. The fact that Figure 26 represents the mean CO_2 production rate of three soil columns is also partially responsible for the smoothing.

Thus it would appear that for the resolution of community components from community activity data to be possible, the community must either be in a stressed condition (resulting in a reduced number of active components) or it must be studied by measuring a parameter which specifies relatively few components.

F (4) Attempted equilibration of a microbial community in the pedostat.

(a) Theory

In water-percolated soil columns, equilibria, if they occur will be of short duration since sooner or later, some nutrient will become limiting as it is leached from the soil. This process will take place progressively with a series of nutrients resulting in qualitative changes in community structure, thus obviating equilibria. A reasonable aim would be to extend the duration of possible equilibria by supplying nutrients which are likely to be limiting. This accepts the inevitability of equilibria of limited duration; however, permanent equilibria would have no counterpart in nature.

The use of a carefully chosen substrate would allow the behaviour of the community to be assessed in terms of two parameters - CO_2 production and substrate disappearance. Thus as well as providing the possibility of equilibrium conditions, such a system would allow correlations of the two parameters to be investigated, allowing assimilation efficiencies and biomass production estimates to be calculated.

The nature of the growth-sustaining nutrient is of great importance in the adoption of this approach. Substrates which may be intermediary metabolites common to a variety of organisms are of no value since they rarely occur free in soil. The substrate should be a simple molecule whose metabolism has been well studied in a variety of organisms. A simple method of quantitative analysis is also desirable. Simple phenolic molecules conform to these requirements and are ecologically relevant substrates. Vanillic acid (3-methoxy-4-hydroxybenzoic acid) was chosen as a possibly suitable substrate; degradation of this comp-

ound requires enzymic processes which are used in lignin and humic acid decomposition - demethoxylation, hydroxylation and dearomatisation (Pratt et al, 1953; Konetzka et al,1957; Trojanowski et al,1966; Cartwright and Smith, 1967). To this extent it can be considered as a model substrate for studies of lignin degradation and since the pathways by which it is degraded are closely linked to those of such better known substrates as benzoic acid and catechol, a wealth of background biochemical information is available e.g. Rogoff, 1961; Kunc and Macura 1966; Dagley, 1967. The importance of the aromatic nucleus in a variety of modern herbicides and pesticides (Walker, 1972) also means that suitable studies of this kind can be of value in terms of eliciting possible pathways of soil detoxification. The decomposition of vanillin and related compounds in soil and by soil isolates has been studied primarily by Kunc (1971a; 1971b; 1971c; 1972). He found that addition of such compounds to the soil resulted in enzyme induction and enrichment of specific types of micro-organisms.

(b) Methods

(i) Assay of vanillic acid

Vanillic acid was estimated colourimetrically after reaction with diazotised p-nitroaniline. p-nitroaniline (0.5% in 2N HCl, 0.5ml) was diazotised immediately prior to use by admixture with 0.5ml 5% aqueous $NaNO_2$ and 1.5ml 20% aqueous CH_3COONa. After mixture of 0.3ml of this reagent with 3.4ml vanillic acid solution, 0.1ml 20% aqueous Na_2CO_3 was added whereupon a maroon colour developed. The absorption spectrum of the coupled diazo-dye exhibited a single broad peak at 508-514nm. The solutions were mixed in a spectrophotometer

cell and examined immediately. Absorption at 512nm was calibrated against vanillic acid concentration and the two were found to be linearly related over the range 0.25-50 nanomoles/ml vanillic acid.

(ii) Extraction of vanillic acid from soil column effluent.

Effluent solution from pedostat soil columns was a deep brown colour. When vanillic acid was added to such a solution and assayed as described above, masking of the colour reaction took place. This problem was avoided by extraction of the vanillic acid with ethyl acetate. The partition of vanillic acid between this solvent and water was such that five successive extractions (1:5 ethyl acetate:water) were sufficient to transfer almost all the acid to the organic solvent. Effluent/ethyl acetate mixtures were separated by passage through Whatman IPS phase separating paper, and the pooled organic extracts were evaporated to dryness at 60^{0}C under reduced pressure. The residue was redissolved in water and assayed for vanillic acid as described above. Recoveries of ca. 95% were obtained of vanillic acid added to effluent liquid.

(iii) Preparation of vanillic acid containing eluents

In order to avoid vanillic acid pedostat cultures becoming simple crude enrichments swamped by the effects of the added substrate, it was necessary to add the acid at a physiologically realistic concentration. At the same time it was desirable that CO_2 derived from the added vanillic acid should make up a significant proportion of the community CO_2 production so that variations in substrate and metabolite concentrations could be compared. Assuming 60% carbon assimilation efficiency (p.157), if all CO_2 produced during soil elution (e.g. 115 nanomoles/g soil/hr - Fig.23) were derived from vanillic acid, the

substrate would represent 0.2% of the native soil organic matter. Vanillic acid was mixed with following mineral salts solution to provide concentrations equivalent to 0.1, 0.05 and 0.025% of the soil organic matter.

Mineral salts (autoclaved, 121°C):

K_2HPO_4 0.5g/litre

$MgSO_4 \cdot 7H_2O$ .1

$(NH_4)_2SO_4$ 1

FeEDTA .06

trace elements 5ml

water to 1 litre, pH to 7

Vanillic acid to 67.5, 33.8 or 16.9 µg/ml (membrane-filtered).

Trace element solution ;

$ZnSO_4 \cdot 7H_2O$ 0.5g (reagent grade)

$MnSO_4 \cdot 3H_2O$ 0.5

$Na_2MoO_4 \cdot 2H_2O$ 0.5

water to 1 litre, acidified with 0.1N H_2SO_4.

Sterile reservoir syringes were filled aseptically with the appropriate media. Five replicate columns were set up for each of the following treatments: (1) eluent water, (2) eluent mineral salts, (3) eluent mineral salts + vanillic acid,0.1% S.O.M. (4) eluent mineral salts + vanillic acid,0.05% S.O.M. , (5) eluent mineral salts + vanillic acid 0.025% S.O.M. (6) control soil-free columns.

(c) Results

Immediately after the standard growth promoting stimulus of soil neutralisation (p.137) had been applied, the pedostat reservoirs were

arranged as described above and the machine was switched to continuous flow. After 80hr had passed, CO_2 production rates and effluent vanillic acid concentrations were determined every 24 hr for 220 hr on two replicates of each eluent variation. Effluent vanillic acid concentration was uniformly zero throughout the experimental period. Mean CO_2 production rate variations are shown in Figure 27. The water eluent treatment columns behaved essentially as expected (Figure 23) while the amended eluent treatments were virtually indistinguishable from one another. There appeared to be a distinct mineral effect in that less regular oscillations of more widely varying amplitude appeared in columns treated with mineral amendments. Since the mineral effect was indistinguishable from the (mineral + vanillic acid) effect, no detectable CO_2 production from vanillic acid took place. This could have been the result of chemical or physical non-availability of the substrate or the absence of a vanillic acid degrading capability in the communiy. The absence of vanillic acid in the effluent suggested that the first possibility was the more likely. Confirmation of the conclusion was gained by incubating γ-radiation sterilised (2.5 Mr) soil columns and unsterile soil columns overnight under saturation with solutions of a range of vanillic acid concentrations.

The sterile soil was capable of absorbing at least 1 micromole of vanillic acid/g soil; this behaviour was indistinguishable from that of the unsterile soil. Experimental vanillic acid concentrations had been maximally 0.85 micromoles/g; thus it was concluded that chemical or physical immobilisation of the added substrate was probably the reason for lack of differential response by the substrate-amended soil columns. Although certain biological activity is retained in soils sterilised by γ-radiation (McLaren et al, 1962; Peterson, 1962;

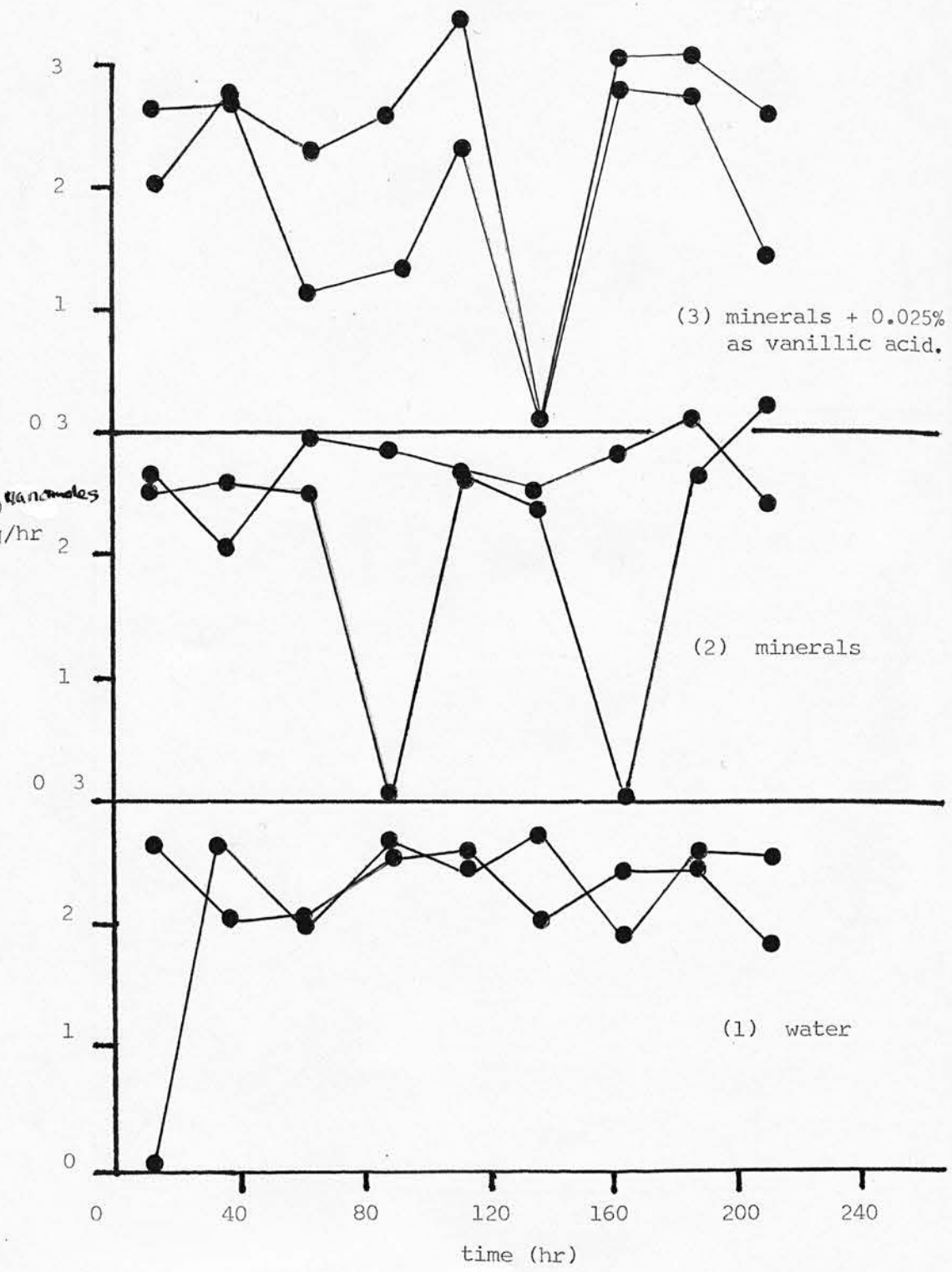


Figure 27.

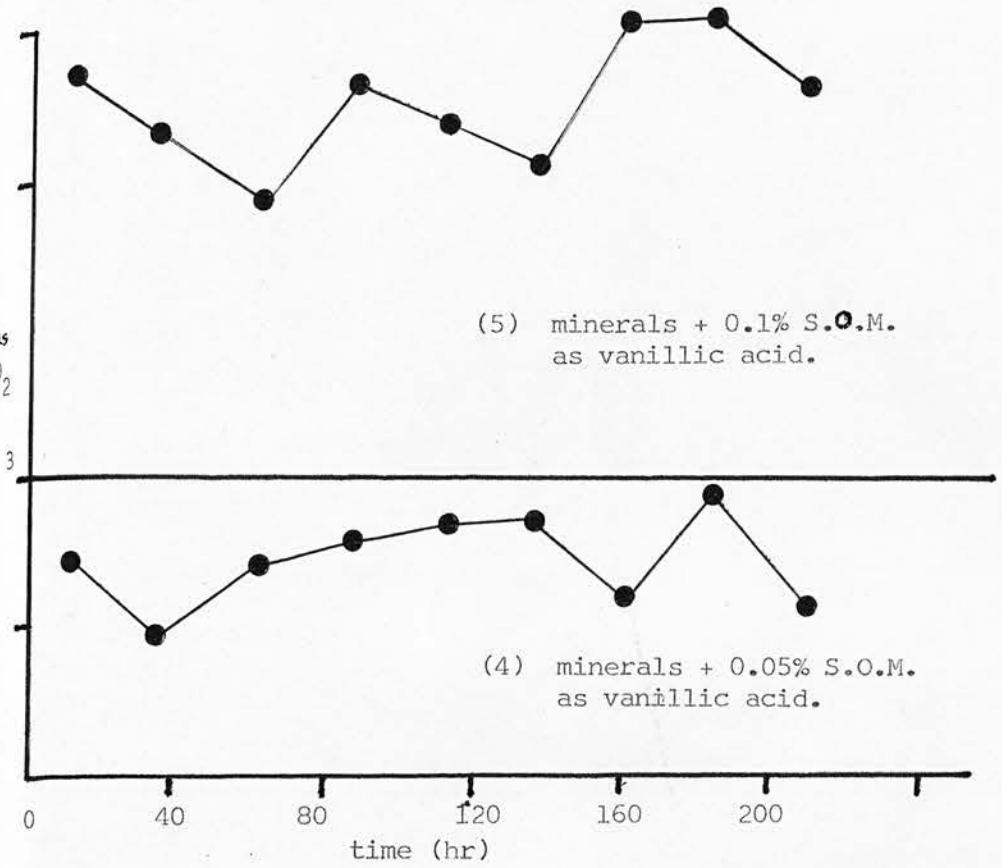


Figure 27. Response of the model soil microflora to elution with water, minerals and vanillic acid. (Where extensive between-replicate phase or amplitude differences occurred, these are indicated - treatments (1), (2) and (3)).

McLaren, 1963; Jackson et al, 1967; Robinson et al, 1971) and some slight physical and chemical modifications of the soil fabric take place (Bowen and Cawse, 1964; Griffiths and Burns, 1968), it was not considered that such factors could exert sufficient effect to invalidate the conclusion.

It is possible that higher concentrations of vanillic acid would have produced a detectable effect once all the absorbing sites on the soil matrix had been saturated. Alternatively, the substrate may have been subject to polymerisation into humic complexes. Thus the interplay of physical, chemical and biological reactions in the model soil is stressed even in such an apparently simple problem as administering a substrate to its microflora.

GENERAL DISCUSSION

(1) Introduction

The defined aims of this study (p.56) were to investigate the applicability of a unitisation hypothesis to soil microbial communities and to assess the adequacy of available techniques for this project. The first (not unexpected) conclusion reached in consideration of the problem was that available techniques and much generally accepted dogma were inadequate. Consequently the bulk of the present work has been directed towards the development of new techniques and equally importantly, new approaches. This procedure ultimately resulted in the elaboration of methods which under suitable conditions could allow the unitisation hypothesis to be tested rigorously. The work stopped short of the testing stage because of the time involved in dealing with the problems encountered in the development of suitable methodology. Consequently, this discussion concentrates on the integration of the findings of this study and their future extension to a stage at which the unitisation hypothesis may be adequately tested.

Before discussing the results it is useful to consider why it has been necessary to formulate an empirical unitisation hypothesis and to test its applicability to soil systems. Some of the intricacies of simple two-species interactions have already been indicated (p.48); the application of theoretically derived mathematical models to soil systems is currently impossible because of (a) the large number of types of micro-organisms present, each one theoretically capable of exerting some

influence on each other one; and (b) the fact that no general conceptual model of the inter-relationships between different types of micro-organisms present in soil exists at present. Thus it is impossible to define a microbial community or to say that specific types of soil micro-organism behave reproducibly in a certain manner with respect to other specified types of micro-organism. In no case is the number of types of micro-organism known and the properties exhibited in the soil by known types of micro-organism are largely a matter of conjecture.

Even the use of such an apparently simple concept as "type of micro-organism" is fraught with difficulties in this context. As used in general microbiology such a concept has definite taxonomic implications, and a "type" of micro-organism is one which fits neatly into an arbitrarily constructed classification designed on some real or assumed phylogenetic basis. It is essentially the classification of a cell, the properties of which are interpolated from the behaviour of a statistical population of such cells. The whole object of such a classification is the identification of a cell and its comparison with other cells. Clearly then, the purpose of the concept of "type" of micro-organism" is (a) the satisfaction of the investigators academic curiosity and (b) the creation of a convenient frame of reference for the communication of microbiological information between investigators.

It is difficult to see the ecological relevance of such a purely taxonomic concept. In ecosystems and communities, the function of the organism is manifested in terms of the behaviour of the

population. In the population ecology of higher plants and animals, monotypic populations may often be considered to have functional status, e.g. the predominant secondary production (grazing) on a heather moor may be due to a (monotypic) population of grouse, sheep or psyllids depending on the degree of man's interference with the habitat. In a microbial analogy to this situation, monotypic populations are difficult to find; reference to the possible structure of a decomposer community as outlined on p. 26 will exemplify this. In this scheme, the broadest taxonomic groupings have been used; from nature however, it is not unusual to isolate a number of taxonomically different micro-organisms capable of performing the same or similar biochemical tasks. Whether under certain conditions these could be regarded as ecologically equivalent "types of micro-organisms" is a problem that has not received attention. More important in the present context is the probability that in a functioning microbial community, populations will not be monotypic. In the execution of a specific biochemical task a variety of organisms, possibly in concert, will combine to take the degradative process to its completion. The fact that coupling between taxonomically different components of such populations is sometimes almost obligate is obvious from the difficulties experienced in separating the components of some mixed cultures isolated from natural environments.

Levels of organisation in biological systems vary from the cellular to the organismic, population, community and ecosystem levels. Most purely microbiological research concentrates upon the cellular level of organisation (which in many micro-organisms

is identical with the organismic level). From what has been said above, the subject of microbial community dynamics must obviously be approached at an appropriate level of organisation. In other words, the behaviour of the whole community is of prime importance. In such a complex system as a soil microbial community, obviously a knowledge of the functioning of the system is of more value than a list of its component parts and their relative concentrations. Unfortunately many soil microbiologists have chosen to attempt to construct such lists of components in terms of taxonomic groups of micro-organisms and their relative preponderance. It has already been indicated that this is a task which is at present impossible to complete (p. 52). Even presented with a complete list of community components, no investigator could be logically expected to be able to predict either the behaviour or structure of the community. Apart from the simple enormity of the task, this would be impossible because any complex system is more than the sum of its parts. This is a well known biological concept (Brock, 1966; Odum, 1969).

Apart from the integrated function of a community its next most important property is its structure. A knowledge of this requires investigation at the population level of organisation.

The complexity of natural microbial systems that has been outlined above and in earlier parts of this work is manifested in terms of interactions between populations, their components, their environment and the community as a whole. It is in precisely this field that knowledge is at its weakest among soil microbiologists. Clearly some logical basis for the analysis of such a complex set

of interactions must be found before experiments on community structure can be adequately planned. It has been suggested (Brock, 1966) that Information Theory may provide the basis for such an analysis. Resumēs of the subject and its application to biological systems have been provided by Quastler (1953; 1958). The subject has not apparently been applied to microbial ecology and the following paragraph is based on Brock's (1966) interpretation of its potential usefulness.

Interaction between organisms requires communication i.e. transfer of information. In biological systems, information may be transmitted by physical or chemical means. It may be contained in the unique spatial configuration of a molecule (e.g. an enzyme or antibiotic) or in a specified sequence of certain repeating elements (e.g. genetic information). The transfer of information may affect a unit in a positive or negative (stimulative or inhibitory) way; if the unit is not affected at all, then by definition, information has not been transferred. Thus information must be equated with the target as well as the element transferred (e.g. the information content of a hormone is not conveyed by its chemical structure alone, but by its structure and the receptive nature of its target). Two units may exchange information in a reciprocal fashion; alternatively transfer may be unidirectional. The coupling between two units may be tight (obligate) or loose (adventitious). According to Quastler (1958) "If two parts are coupled in any fashion, then knowledge of the state of one must imply some information about the state of the other." In

microbial communities, it has already been noted (p. 51). that complete coupling among all units is unlikely because of its inherent inflexibility. According to Quastler (loc.cit.) "It is an empirical fact that when a system is complex enough to require many components, the phenomenon of unitisation occurs. That is, some components become organised in such a way that they interact strongly among each other and act as a unit with respect to the remainder of the system ..... Unitisation is always coupled with the phenomenon of limited span. Any real part has a limited information content. In any single act of communication, the capacity for non-redundant transmission of a part is limited by its own information content. This amount must somehow be partitioned into interaction with the external world, and interaction with other members of the unit. If each of these interactions is to be of significant size then only a limited number is possible. The interaction of a unit with the outside may be only a fraction of the information traffic within the unit. Hence several units may be organised into a secondary structure of greater versatility, and this process can be repeated on successive levels of organisation."

It is emphasised that this type of description of a complex system is purely empirical and is based on the consideration of a large number of analogous biological systems. However, the analysis of new problems by reference to isomorphic situations is a standard mathematical procedure. The term "unit", although having a constant meaning in the abstract form may be applied to components of varying complexity at levels of organisation

varying from the cellular to the ecosystem. Clearly, unitisation allows an enormous simplification of the analysis of microbial communities. Instead of the thousands of millions of individuals comprising hundreds or thousands of taxonomic types per gram of soil, the situation has been reduced to a limited number of functional components - population units which are ecological rather than taxonomic in their definition. Adoption of this approach allows the elaboration of a conceptual model of a soil microbial community. The previous absence of such conceptual models has been stressed above (p. 176).

Thus the community, which is itself a unit in the ecosystem is made up of population units whose structure is such that within themselves they are probably fairly tightly coupled in comparison with between population-unit interactions. Any system which exhibits tight coupling possesses certain integrative properties which are useful in characterising and analysing the system (Brock, 1966). These may be listed as homeostasis, evolution, defence, repair and reproduction. Use of the term homeostasis does not imply that the system is not affected by external influences but that when subjected to them, it is able to retain its integrity. Evolution can be regarded as a long-term modification of the homeostatic structural equilibrium in accordance with sustained environmental stimuli. Defence is specifically a response to adverse environmental stimuli and the rejection of 'alien' micro-organisms introduced into an established system has already been noted in this context (p. 60). Repair of a part of the system takes place after damage as a mechanism of homeostasis.

Reproduction is manifested as the complete replacement of a system by another one which is similar or identical. In a microbial system with relatively very short generation-times, reproduction must be closely linked to homeostasis, i.e. in the face of a constant environment proliferation of the system is probably self-limited. The above integrative properties are exhibited by cells, organisms, populations, communities and ecosystems although the coupling between the elements of the unit (hence the degree of development of the integrative properties) obviously decrease in tightness from the cellular to the ecosystem level of organisation. The fact that coupling exists in microbial systems is shown by the already noted fact that it is often difficult to isolate in pure culture organisms which reproduce readily in mixed culture e.g. methane producing anaerobes.

(2) Experimental verification of the unitisation hypothesis

Problems in the choice of appropriate community parameters (p.33 and 79) have usually led to soil microbiologists' choosing the easiest and least satisfactory method of analysis, the plate count procedure. Theoretically it would seem to be possible using an ideal plate-count procedure to isolate a group of taxonomic types of micro-organisms whose proportions one to another remained constant while the group as a whole varied quantitatively with respect to the rest of the microflora. The existence of such groups of organisms could be taken as presumptive evidence of the occurrence of unitisation. Practically however, such an approach is clearly impossible. Not only does it require

"ideal" plate-counting conditions - an impossibility but also it presumes some knowledge of the taxonomic composition of a unit; i.e. in the absence of information on which type of micro-organism to count, it would be impossible to compare differential counts of all colony types which grew on the plates since only the numerically dominant component of the plate-count would be adequately sampled. Although the requirement (above) that unit components should exist in constant proportions may seem at first sight reasonable, it is obviously invalid since it requires that all unit components have the same growth and senescence rate. It also ignores the fact that tightly coupled interactions between organisms often result in oscillatory changes in cell numbers (p. 44) with the numbers of the interacting organisms out of phase with and hence varying with respect to one another. In other words, as well as a knowledge of unit taxonomic structure, some information on the dynamics of unit components would be needed before a group of micro-organisms could be identified as a unit. Many of the drawbacks outlined above apply equally well to microscopic total count procedures; the main advantage of such procedures, their relative lack of specificity is in these circumstances a disadvantage. Thus the most direct means of estimating microbial biomass is ruled out in a study of microbial unitisation. This does not imply that careful use of biomass estimates is invalid in the study of other aspects of (e.g.total) community dynamics.

Since enumerative community parameters are obviously inadequate, the only other possible approach to the problem is to measure some

growth - or activity - correlated metabolic parameter. Since the unitisation hypothesis has been formulated in terms of community function and the functional role of micro-organisms in the decomposer community has been stressed throughout this study, it is perhaps more appropriate to consider a metabolic parameter than an enumerative one. The fact that cell numbers and biomass demanded first attention in the consideration of a suitable community parameter reflects the effect of the momentum of "standard" procedures in soil microbiology. Also from imagined analogies with the study of population dynamics of higher plants and animals, enumerative procedures acquire a spurious authenticity. The limited analogy to such systems is manifested mainly in the multifunctionality and variety of micro-organisms when compared to higher organisms and in the dubious importance of the monotypic population. The availability of so many micro-habitats in soil makes it possible for a wide range of micro-organisms to be accommodated in a very small sample of soil. Since the mobility of micro-organisms is relatively restricted this adds to the difficulty experienced in comparing microbial systems to higher plant and animal systems.

The use of directly growth-correlated metabolic parameters (i.e. quantitative analyses for some cellular component) has received some attention recently. Thus Miller and Casida (1970a,b) developed a procedure for extracting muramic acid from soil and sought to relate concentrations of the compound to bacterial biomass. The method has not received wide application probably because of one main defect: muramic acid content varies between different

types of bacteria e.g. 4 ng/10^6 gram positive bacteria, 0.5ng/10^6 gram negative bacteria and 66ng/10^6 aerobic bacterial spores. The criticisms levelled by Parkinson, Gray and Williams (1971) that the method is incompatible with the plate count are not valid since they imply faith in the validity of the plate count. Working on analogous lines Swift and Nesbitt (1970) have attempted to estimate soil fungal biomass and production.

An alternative partly growth-correlated and partly activity-correlated parameter is soil adenosine triphosphate level (e.g. Doxtader, 1969). The method was originally developed as an extraterrestrial life detection technique. Its attractiveness however appears to be in the elegance of the method of ATP quantitation used: a photometric assay based on light emission when ATP is linked to a firefly luciferin/luciferase system. ATP content g/g dry weight of a variety of soil micro-organisms has been tabulated by Ausmus (1973); values from .0006 to 0014 were noted for most bacteria while values for fungi and actinomycetes were similar, ranging from 0.0016 to 0.0028. Differences in cellular ATP pool size occur depending on the growth phase of the organisms and the compound is presumed to be absent from non-living cells. Despite the many difficulties obviously involved in the application and interpretation of this method, it may hold promise for future use.
Its drawbacks have been discussed earlier (p.80).

All enumerative and chemical analyses discussed so far necessitate destruction of the sample. In view of the limited synchrony of

replicate soil cultures (p. 139 and Fig 27) sample destruction was not desirable and some means of continuously monitoring an activity-correlated parameter on an intact sample was clearly the optimal method. In this light, monitoring the production of a volatile metabolite which could be removed without damaging the sample was attractive. Since it has been stressed that catabolic activity is likely to be an ecologically relevant attribute (p.111) CO_2 production was the obvious choice for continuous monitoring. A large variety of methods of measuring CO_2 production have been used in studies primarily on decomposition (e.g. Chase and Gray, 1953; Rovira, 1953; Smith and Brown, 1932; Wallis and Wilde, 1957; Drobnik, 1958; 1960a,b; Gilmour et al, 1958; Stotzky, 1965; Witkamp, 1966; Stout and Dutch, 1968; Macfadyen, 1970; Tomašek and Knotkovā, 1970 and many others). Although a significant correlation between CO_2 production and bacterial numbers in soil has been noted (Gray and Wallace, 1957), little if any use of CO_2 production data has been made in the study of microbial dynamics in soil.

It was however noted by Lemmerman and Weissman (1924) that CO_2 evolution from organic matter in incubated soil appeared to be related to time by the equation

$$X = a.k.t^m \qquad (20)$$

Where 'X' was the total amount of CO_2 produced in time 't', 'a' was the carbon content of the soil at the beginning of the experiment and 'k' and 'm' were constants. They showed that when log X was plotted against log t a linear relationship was obtained

over their experimental period of 995 days; addition of hay or straw to the soil caused disturbances for only the first 15-30 days. Corbet (1931, 1934) modified equation (20) to

$$Y = F\, t^{m} \qquad (21)$$

where $Y = X$ and $F = ak$. He considered 'F' as a fertility constant referring to properties of the soil while the constant 'm', a measure of the constant rate of decrease in CO_2 production was considered to be dependent on laboratory conditions. Corbet (loc. cit.) found that this equation fitted his experimental data on CO_2 evolution from some Malayan plantation soils. Millar, Smith and Brown (1936) also confirmed the applicability of Corbet's equation (21) in experimental studies lasting up to 280 days. Chase and Gray (1957) converted Corbet's equation to a differential form and studied regressions of log O_2 uptake against log t instead of cumulative CO_2 production. Again, after an initial adjustment period, the results confirmed the validity of Lemmerman and Weissman's original empirical description of soil CO_2 production.

Chase and Gray (loc.cit.) carried out a fascinating examination of the significance of the linear log rate - log time function; in this, although using different terms, they essentially antedated Brock's (1966) suggestion that a unitisation hypothesis may be applicable to complex microbial systems. Chase and Gray approached the problem with the knowledge that virtually any laboratory manipulation of a soil sample (e.g. drying, moistening, disruption, etc.) results in an immediate increase in the amount

of readily-degradable water-soluble organic material (Gustafson, 1922; Khalil, 1929; Lebedjantser, 1924). During the laboratory incubation of a soil sample, they assumed that this readily degradable substrate was available to the microflora at the same time as a more slowly degradable humic component of the soil organic matter. At this point, they introduced Winogradsky's (1924) principle of autochthonous and zymogenous groups of micro-organisms. They supposed the two groups of organisms to be functioning independently, each upon its selected substrate according to the exponential or first order reaction law, with the concentration of substrate being ultimately the limiting factor. From this reasoning, it is clear that at least two different first order reactions would be superimposed one upon the other, with the result that the combined values, if plotted log rate against linear time, could not be expected to yield a straight line, but rather a curve with at least two segments. Thus plotted, data from experiments carried out by Chase and Gray showed a definite tendency to give a graph with an initial curve followed by a straight line. This is illustrated in Figure 28. Assuming that the second or straight line portion of the curve was caused by the breakdown of humus, Chase and Gray projected the straight line backwards on the premise that humus was being broken down in accordance with this straight line function since the start of the experiment. It was then possible to calculate by difference the rate of oxygen uptake accountable each day for the oxidation of the more readily degradable organic fraction. When the logarithms of these daily rates were plotted against time, a good fit to a second straight line was obtained. Chase and Gray

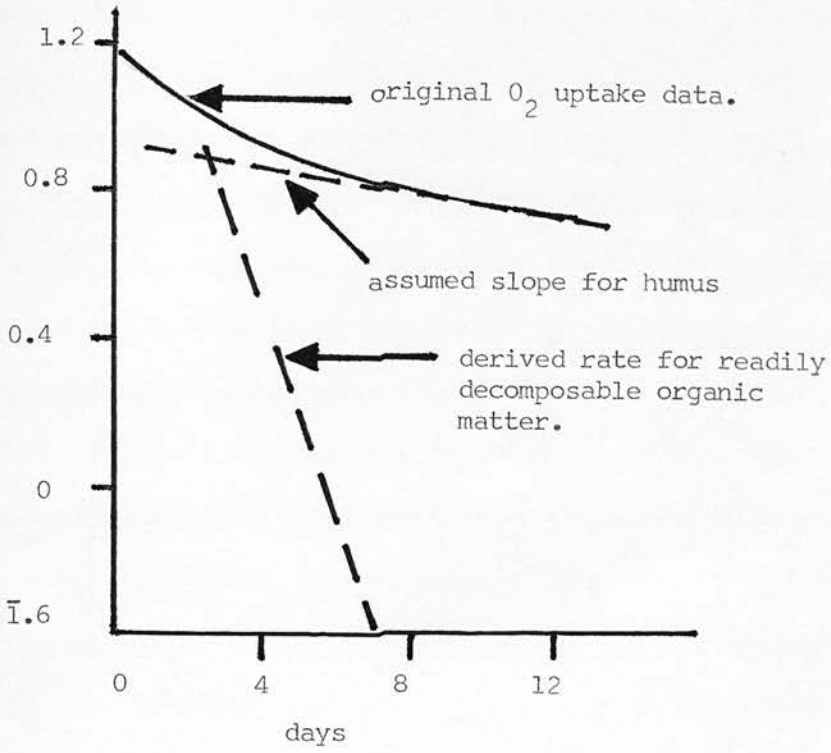


Figure 28. Chase and Gray's (1957) derivation of the rates of decomposition of 'humus' and readily decomposable organic matter.

concluded that the linear log rate - log time function which appeared to provide a good empirical description of soil respiration data may arise from a situation where two or more first order reactions are progressing simultaneously. They recognised that there are probably a number of components of native soil organic matter which conceivably serve as substrates in a whole series of super-imposed first-order reactions each with distinctive reaction velocities. They concluded that the fact that the soil organic matter in their experiments appeared to be made up of a simple two-component complex suggested that the reaction velocities of the two types of substrate were large in comparison with those of residual organic matter components; the latter substrates could only be expected to become dominant (hence detectable) after the more readily oxidisable fractions had become partially depleted.

In essence, this interpretation of soil respirometric data is equivalent to the formulation of the unitisation hypothesis. The difference lies in the fact that the unitisation hypothesis was derived empirically by analogy with isomorphic situations and Chase and Gray's hypothesis was derived empirically on the basis of experimental data. The fact that Chase and Gray succeeded in resolving two community components which could justifiably be called units cannot be regarded as evidence for their hypothesis, rather this demonstrates its internal consistency. The resolution of the two components is however in agreement with the less mechanistic unitisation hypothesis and emphasises the fact noted above that this must at least initially be formulated in functional rather than structural terms. Thus resolution of the zymogenous

and autochthonous components necessitates the assumption that their activity in soil is independent. There has been little experimental work on this subject; however on agar plates, inhibition of presumed autochthonous organisms by rapidly growing (hence zymogenous) types is part of the accepted dogma (e.g. Topper, 1968).

Unfortunately, none of the authors quoted above who applied Lemmerman and Weissman's equation to soil respiration data apparently attached any significance to the pattern of variation in daily CO_2 production rates. This is understandable since most of the experiments carried out were long-term and variations which are large in short-term experiments may be small compared to the drop in mean rate over a longer time period. Consequently, none of the authors described the oscillatory pattern of activity noted in this study. The experiments carried out corresponded to point stimulus studies with extended incubation periods. The growth-promoting stimuli were simply the manipulation of the samples, sustained activity being a function of the momentum of the community. The point-stimulus experiment described in this study was not extended for a long enough time to allow comparison with the experiments described above. The water-eluent pedostat experiment (p.160) did however provide sufficient data. If a log CO_2 production - log time plot of Figure 23 is made, it can be seen (Figure 29) that after a short initial 'adjustment' period activity conforms to Lemmerman and Weissman's equation. The linearity of the terminal portion of the curve shows clearly that activity is declining uniformly rather than approaching equilibrium (p.162). The initial 'adjustment period' is of interest in terms

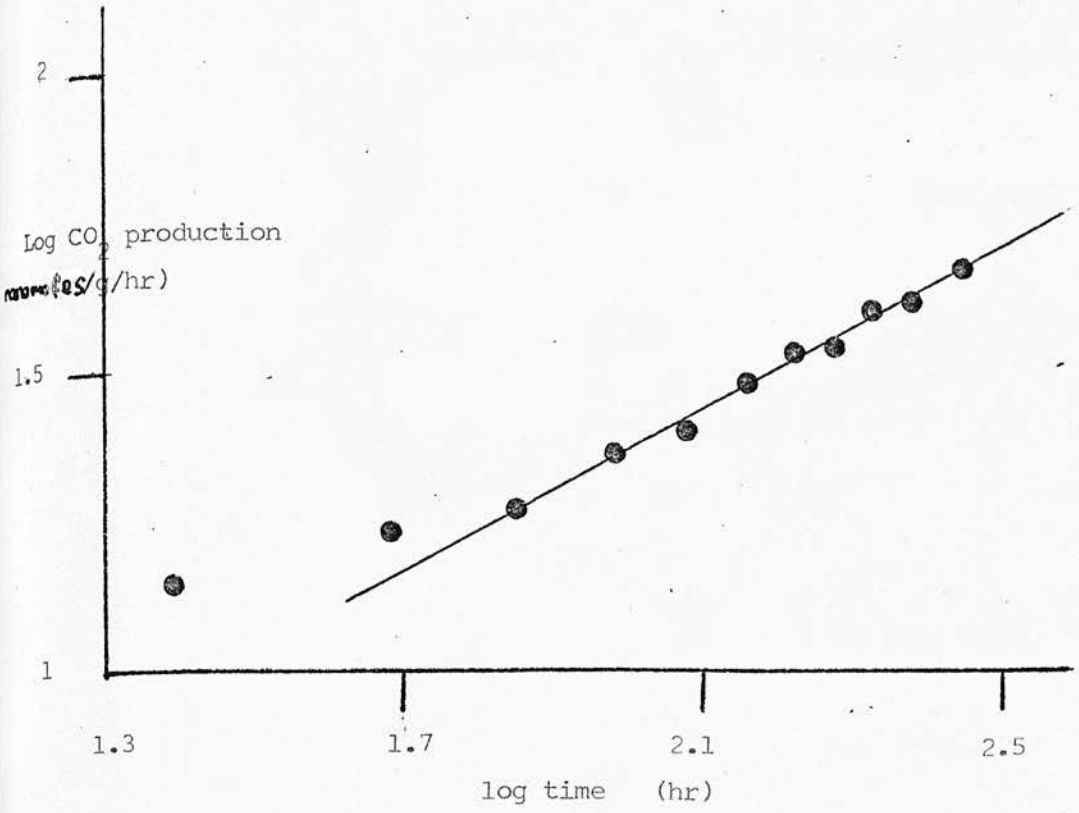


Figure 29. Log rate/log time plot of Fig. 23 (continuously stimulated activity portion).

of Chase and Gray's analysis. Because of the observed oscillations in log CO_2 production rate vs linear time (Figure 23), an analogue of Figure 28 must be drawn using gradients from Figure 25, the cumulative CO_2 production curve. This effectively smooths out the oscillations giving a mean net CO_2 production rate curve (Figure 30), upper portion. A difference from Figure 28 is immediately obvious: latterly the change in 'humus component' CO_2 production rate appears to be zero. (i.e. in real terms a steady production of ca. 0.15 $\overset{\text{nanomoles}}{\wedge}$ CO_2/g/hr appears to result from the degradation of the humus component of the soil organic matter). By subtraction, the exponentially declining rate of CO_2 production resulting from the 'readily degradable' component of the soil organic matter was obtained. Although two components have thus been resolved, it is not warranted to calculate half-lives for the two chemical components of the soil organic matter in the way that Chase and Gray did, since the smoothing involved in arriving at Figure 30 has introduced too many inaccuracies into the slopes of the straight lines representing the two components. This is clear from the fact that Figure 30 indicates a stabilised terminal CO_2 production rate of 150 $\overset{\text{nanomoles}}{\wedge}$/g/hr derived solely from 'humus' while the experimentally determined data (Figure 23) indicates that the rate of CO_2 production is terminally still declining and oscillating between 60 and 250 $\overset{\text{nanomoles}}{\wedge}$/g/hr.

It is interesting to note in this context the one case of non-oscillatory activity observed in this study. This situation occurred when alongside neutralised soil columns, three columns which had been treated simply with water as a growth promoting

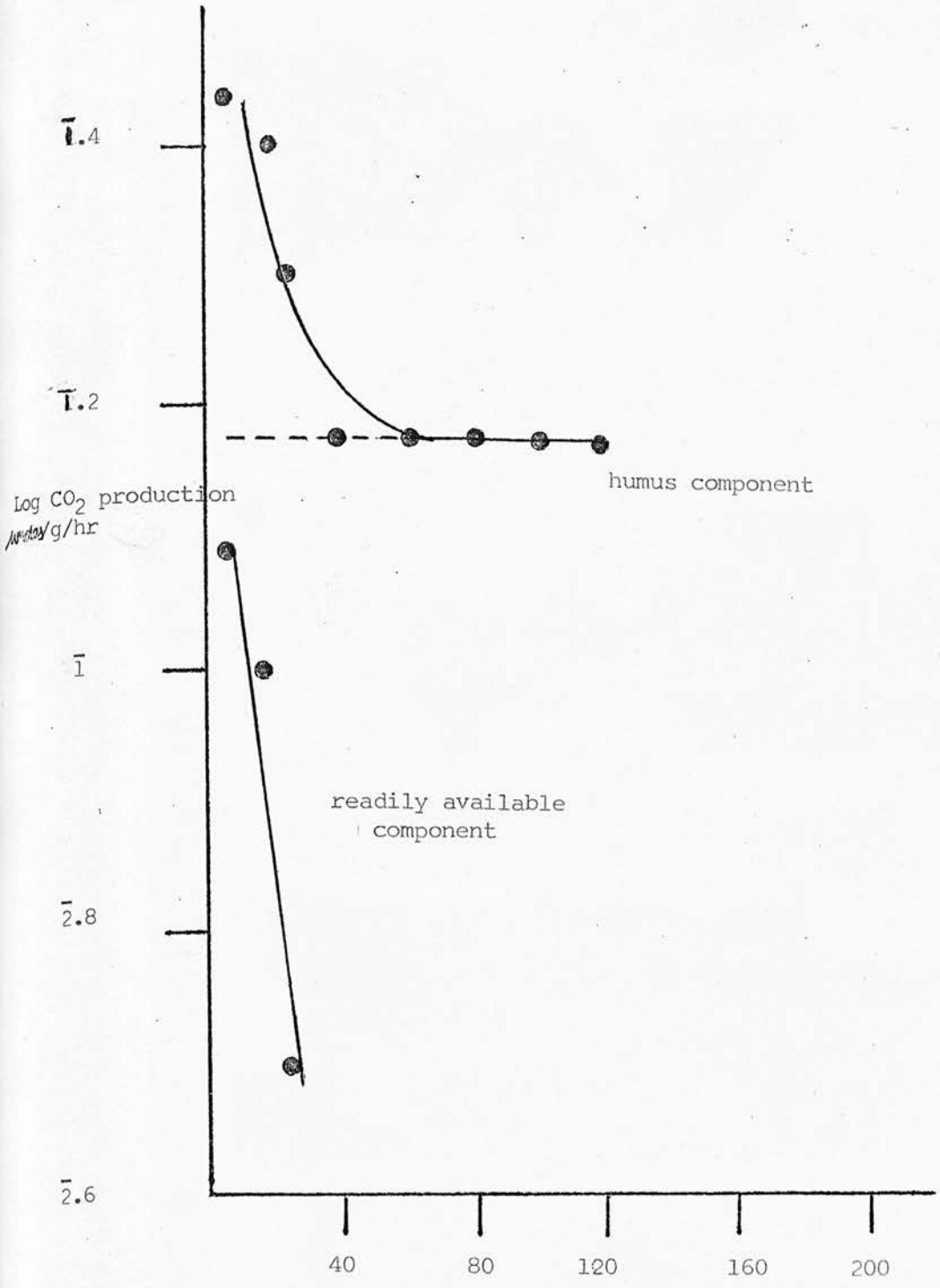


Figure 30. Log rate/linear time plot of the gradients of Fig. 23 (cumulative CO_2 production).

stimulus were observed. The pH of the soil in these columns did not differ significantly from that in the original soil. Between 132 and 156 hours after the start of the experiment, mean CO_2 production rate fell below the limits of detection. A plot of log rate vs linear time showed a smooth curve with no linear tail. This may be interpreted as the result of activity being almost completely due to nutrients made available by the addition of water; background 'autochthonous' activity was so small as to be below the detectable limit. Such a situation is entirely in accord with expectations in such an acid soil. When however, a graph of log rate vs log time was plotted up to 156 hours (Figure 31) it was found that the data conformed largely to Lemmerman and Weissman's description.

Thus it appears that although Chase and Gray's analysis was useful in providing presumptive evidence of unitisation, its quantitative application to oscillating systems is unsatisfactory. It is probable that it would have been more applicable had Figure 23 been carefully smoothed by taking its mean at intervals along the time axis. The results obtained would however be too idealised and divorced from reality; the best way of applying Chase and Gray's approach would be to study a comparitively long-term experiment (months rather than weeks) where for the reasons noted above oscillatory behaviour may not be significant. However, as noted earlier, such a procedure could not provide a realistic comparison with a natural soil since it would be essentially the study of a declining closed system. The fact that gross pH adjustment is a rather extreme growth-promoting stimulus should not

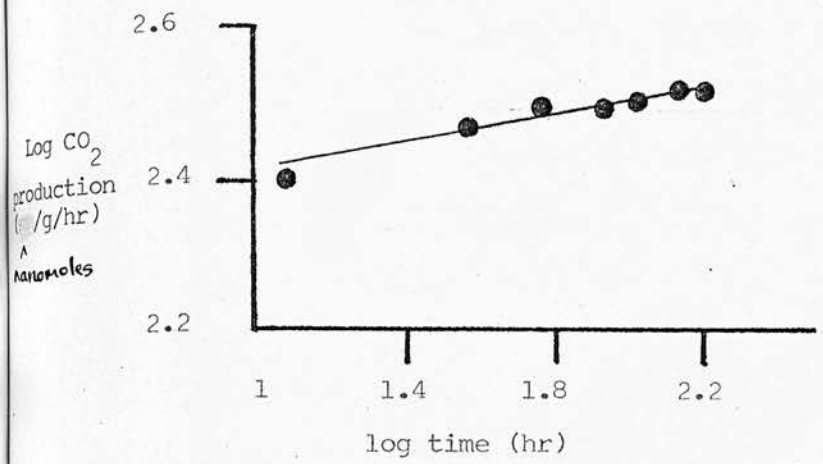


Figure 31. Lemmerman and Weissman plot of the activity data from an un-neutralised model soil.

be ignored; more gently stimulated systems would probably fail to oscillate detectably and hence be more susceptible to this type of analysis.

Because of these difficulties, another method was sought to allow the resolution of population units from community activity data. Clearly a method which took account of the oscillatory nature of the community would be of greater value than one that was hindered by it. Although it may be possible to analyse a series of convergent oscillations such as those shown in Figure 23, it would be much simpler if the system were at a steady state. A constraint placed upon a community resulting in its components oscillating at constant (different) amplitudes and frequencies could be interpreted as a stress tending to simplify the system by reducing the number of active components. Any simple modification of pedostat eluents towards this end would take this form since their growth-sustaining effect would be selective. The attempt described (p.169) to stabilise community activity by the addition of vanillic acid failed for technical reasons; had it succeeded it could be expected that the model soil would have become enriched in vanillic acid utilising micro-organisms and their associated unit components. In other words, a 'vanillic acid degrading unit' would have been selected. Again this procedure would not be realistic; a more natural model would probably be to add a soil extract in the eluent. However, the potentially simplifying effect of the vanillic acid solution was a desired feature and to this end its use was justified.

In the absence of steady state community activity data it is difficult to elaborate more than an outline of the projected method of unit resolution. The basic principles of the method have already been discussed (p.164); their application is now briefly considered. It has been noted above that breakdown of specific substrates may be effected by different groups of micro-organisms acting to some extent independently. From the point-stimulus and pedostat experiments it seems likely that the growth and activity of such groups of micro-organisms may be oscillatory under certain conditions. If experimentally estimated rates of CO_2 production were taken as the parameter defining the oscillations, it could be reasonably expected that a composite community oscillation would be the sum of the oscillations resulting from the activity of different groups of micro-organisms. In this way the community activity curve could be considered as a composite wave-form and could be analysed as such, allowing all unit components which made a significant contribution to the curve to be resolved. The sensitivity of the CO_2 production rate estimations would obviously regulate the number of components which could be resolved; for this reason, sampling should be carried out much more often than in the pedostat experiments described.

The shape of the CO_2 production rate wave-form is of importance in any analysis attempted along the lines described above. From the point stimulus experiment, it appears likely that it would more closely approach a saw-tooth function than a sine wave. Likely shapes for such functions would have to be investigated

experimentally using pure and controlled mixed cultures in soil. Although the construction of model mixed cultures was initiated by the preparation of genetically labelled organisms (Section A) for technical reasons this part of the study proved abortive and was discontinued.

In allowing a resolution of population units from a community an analytical system would provide a unique characterisation of that community in terms of the parameter defining the oscillations. This characterisation would be valid throughout the course of growth and decline of the various community components and could thus be considered as a dynamic fingerprint (p.149) of the community. Such characterisations would allow simple comparison between communities and because of the specificity and bias of any single parameter applied to such a complex system, would probably allow quite a sensitive differentiation between communities. Because of the bias inherent in any single parameter it would be necessary to measure as many activity-correlated parameters as possible to allow aprogressively more complex composite picture of the community to be assembled. This aspect of the study has already been discussed (p.165) and need not be repeated. It is useful to point out however that although steady state communities in replicate pedostat soil samples may be out of phase with one another, similar oscillations should be resolvable from each replicate. Thus a combination of continuous monitoring of one parameter and application of destructive analyses at chosen times would be theoretically possible.

(3) Possible extensions of methods developed in this study

(a) The specific rational count procedure

This term is now used to refer to the method outlined in principle in Section A for estimating the total number of actively dividing cells in a soil sample. Since the experimental model constructed in an attempt to evaluate the method was completely non-functional, future application of the model is zero. Thus the usefulness of the specific rational count method is unknown. Before it could be used on real or laboratory incubated soil samples, it must be rigorously tested by the use of very carefully controlled mixed cultures. The principle of using genetically labelled organisms for this purpose remains valid as does its extension to prepare a range of interacting pairs or groups of organisms (p. 77) for the study of specified types of interaction under controlled conditions. Retrospectively, since genetically 'labelled' organisms are readily available, genetic manipulations by the soil microbiologist are probably best avoided as much as possible. It is too easy for the construction of a technique of possibly minor application to assume the proportions of an entire research project.

In the application of the specific rational count to real or complex model soil systems, the selection of a well-defined indicator component (to provide the V-estimate) is essential. Under suitable conditions this could be a plate-count made on a highly selective medium designed to enumerate a specific type of micro-organism. The accuracy of such a cultural procedure would have to be tested by the recovery of known numbers of the micro-

organisms from a soil previously inoculated. An alternative procedure for the V-component would be to use a series of biomass estimates based on carbon assimilation efficiency experiments carried out in a pedostat. Carefully correlated changes in effluent substrate concentration and CO_2 production could allow biomass estimates to be made in the manner described earlier (p. 165).

(b) The enzymatically active biomass procedure

Apart from the drawbacks associated with any microscopic enumerative method, two related problems were encountered in the use of this procedure: (1) lack of sufficient colour intensity in the formazan; (2) masking and confusing of stained bacteria with inert particles. These problems were mainly experienced in the model soil which had 16% organic matter. In the (more predominantly mineral) garden soil tested discrimination was sufficient to allow adequate counting and the method as described, appears to be applicable to such soils. For more universal applicability however, it is obvious that two major modifications of the procedure are necessary. First, a tetrazolium salt which provides greater contrast between formazan and soil should be sought or prepared. A formazan which allowed transmission of light at the red end of the spectrum would obviously provide greater discrimination than the blue-black diformazan used; NBT formazan (p. 117) was selected primarily on the basis of staining intensity rather than discriminatory power. Red formazans are produced by the reduction of monotetrazolium salts and it is possible that attention could profitably be directed towards this type of artificial electron acceptor.

The second major modification must be in the extraction procedure used to separate the microflora from the bulk of the soil. The inadequacy of extraction procedures is a serious problem in all branches of quantitative soil microbiology. The fact that it has not been possible to supercede the traditional method of mixing soil with water and preferentially diluting out the larger soil particles indicates that any suggested improvements on the technique are conjectural. However, this is a problem which deserves more attention than it has received, so a little informed conjecture is not inappropriate. Suspension of soil in water is usually regarded as the first and final stage in the extraction of soil bacteria; although physical extraction procedures have been used for fungi (e.g. Lisina-Kubik, 1970) and protozoa (Heal, 1971) these are not applicable to most bacteria. The preparation of a suspension and sedimentation of the larger components should be regarded as a necessary first stage in an extraction and purification procedure. At this stage, the use of appropriate detergents allows the separation of a supernatant containing single cells, clumps of cells and soil particles of microbial dimensions. Physical or chemical methods of disaggregating the clumps at this stage would be appropriate; these could take the form of surface-active agents or possibly protease or carbohydrase preparations.

After this stage, a physical method of separating animate from inanimate objects is required. The property of cells upon which this discrimination may be based is unlikely to be involved in any direct demonstration of vitality. It would be more appropriate to look for a purely physical discriminatory criterion. For this

reason, use of the cellular surface charge offers considerable promise. Fractionation of micro-organisms on this basis by the use of ion-exchange columns is not attractive because of the probable elaboration of further, less specific solid surface/micro-organism bonds when the adsorbant comes into contact with the microflora. For this reason, the new technique of free-flow particle electrophoresis (Strickler, 1967) offers great promise. In this process a thin rectangular film of buffer (electrolyte) is pumped slowly between electrodes which form the long axis of the rectangle. A suspension of particles is injected into the centre of the film as it enters the space between the electrodes. A suitable (adjustable) potential maintained between the electrodes allows particles to segregate differentially according to their charges. Since the film is continuously moving, this results in a linear fractionation of particles parallel to the applied field. The particles may then be harvested according to their electrophoretic mobility at suitable points along the film's line of exit from the space between the electrodes. Apparatus exists which allows continuous injection of samples and continuous harvesting of fractions. Adjustment of the composition of the buffer would alter the ionisation of cell surfaces and coupled with field and flow-rate alterations allows the selection of a wide range of sensitivities. The method has already been used to separate simple mixtures of bacteria and solids; to the author's knowledge, it has not been applied to soil suspensions.

Because of the difference in charge patterns between clay particles, organic material and micro-organisms, it is possible that the

electrophoretic method would be at least partially successful. Since effluent from the various sampling ports could be simply concentrated by centrifugation or membrane filtration, sufficient material for a variety of chemical, biochemical or physical analyses could be obtained. By suitable adjustments of the conditions, it may be possible to isolate organisms in a state very similar to that in which they existed in the soil - an achievement which has been to date impossible. Further fractionation of partly purified effluents could be achieved by repeated electrophoresis under different conditions, density gradient centrifugation or cultural means. The applicability of density gradient centrifugation techniques in the separation of mixtures of micro-organisms is well known (e.g. Mitchison and Vincent, 1965; Juhos 1966). The fact of having physically fractionated microflora components would allow community fingerprints (p.149) to be constructed on the basis of chosen taxonomic, biochemical or physical parameters.

It could be anticipated that application of the enzymatically active biomass procedure to purified suspensions would present fewer problems than its application to cruder suspensions. However, in any extended usage of microscopic counting and measuring procedures, the very significant time and effort expended upon the methods become a limitation to their efficiency. For this reason, cell counting and sizing methods must become automated. The use of the Coulter counter would be feasible only with very clean preparations. A technique which offers more promise is the use of direct microscopy coupled with an image analysing computer facility. Apparatus and techniques for such

analysis exist and are used in branches of medicine, minerology and marine microbiology; again however, to the author's knowledge, they have not been applied to soil suspensions. The apparatus consists of a standard microscope equipped with a television camera. Visual information from the microscopic preparation is monitored on a cathode ray tube and converted to electrical information to be fed to appropriate logic circuitry. The computer component can be programmed to count cells in selected fields, to sort the counts into those related to size categories of cells, to measure the visualised cells and to calculate the numerical and volumetric distributions in a statistically valid manner. The speed of these operations is such that numerically minor community components could be adequately sampled. Differentiation between micro-organisms and inanimate objects could be made on an objective basis and the sensitivity of the differentiation could be altered in a controlled manner. Theoretically, this could allow a discrimination between numbers and biomass derived from enumerative data.

(c) Improvement of the pedostat

For the purpose described (p. 164) the major modification required to the pedostat is the provision of some facility allowing high frequency of sampling of effluent gas. Provisionally, this should be arranged on an hourly basis. The number of alkali samples which this would provide for analysis would be prodigious for any reasonably extensive experiment. Consequently, some automated means of analysis is desirable. The described chromatographic analysis (p. 223) coupled with an arrangement for automatic gas

sampling would be adequate for this. A means of collecting all the air passed over (see below) or through soil samples could be allied with very low aeration rates and the abolition of alkali CO_2 traps. This could be accomplished by completely flushing the air space associated with a soil column with a larger volume of CO_2-free air, then introducing a sample of the effluent gas into the gas chromatograph. This could be arranged so that columns were flushed sequentially and a number of replicate samples of gas analysed if required. In this way the air-supply system could be incorporated into the pedostat. Alternative provision for the attachment of standard alkali CO_2-traps would increase the versatility of the machine by allowing concentration of $C^{14}O_2$ in studies of the degradation of radioactively labelled substrates.

For preference, soil samples should be larger than those used at present. This would diminish the importance of edge-effect flow heterogeneity. The soil should also be packed onto a tension table or sand layer of known porosity to allow its maintenance at a known pF gradient. This procedure would present only one soil/air interface thus flushing air would be passed over the soil surface rather than through the sample. This arrangement would be more natural and could be expected to reduce between-column physical heterogeneity. The effects of forced aeration in model culture systems are felt primarily in modification of the CO_2 concentration in the soil atmosphere. Such effects are well known (e.g. Gaffney, 1965) but were not relevant in the present study since the stage of sophistication of the model soil used did not warrant their consideration.

Finally, the incorporation of soil temperature control into the machine would allow it to be used on a laboratory bench rather than in a constant temperature room. This could be simply provided by the use of a water jacket penetrated by flexible polythene sleeves through which the soil columns would project. Thus the soil columns would be separated from the water by a film of polythene and could be removed and replaced at will while the water was retained in its sealed system. Water could be circulated through the jacket from a thermostated reservoir equipped with heating, refrigeration and temperature programming facilities. In this way, realistic operating temperatures could be simply obtained and the effect of diurnal temperature fluctuation cycles investigated if required.

(4) A conceptual model of the dynamics of soil microbial communities

As discussed earlier, (p.61) one of the formalised stages of systems analysis as currently applied to ecology is the construction after preliminary observations, of a word model of the system under study. The word model embodies what are thought to be the controlling principles and environmental parameters affecting the types of organisms comprising the system. Such a model exposes conceptual weaknesses, provides a basis for the planning of experiments and acts as a framework around which quantitative mathematical models and computer simulations may be constructed. The following word model is proposed as an integration of the findings of the present study along with selected material from the literature which has already been discussed.

(1) The soil environment of the microflora is a three phase system in which the solid phase is porous; the relative volumes of pore space occupied by the gaseous and liquid phases depend on soil structure, climatic and drainage factors. In the model soil constructed in this study, 84% by weight of the solid phase was inorganic material. There was a total of 2.11 ml pore space per g of the solid phase. At 100 cm suction, all pores > ca. 30 μm diameter were drained, the air-filled pore-space being 1.18 ml/g. Based on an experimentally determined mean cell volume of 4.5 μm^3 the water-filled pore space could accommodate maximally 3×10^{10} cells/g in excess of those present (2×10^9/g).

(2) The organic substrates entering the soil system comprise the entire range of biochemicals elaborated by the canopy and the decomposer community. Such substrates enter the soil as solids or aqueous solutions depending on their origin; their availability to the microflora is governed primarily by their chemical nature and secondarily by the physical nature of the soil matrix. Carbon mineralisation is ultimately effected by microbial catabolism. From the limited evidence available, carbon assimilation efficiency in soil appears to be similar to that of *in vitro* microbial systems. Major sources of organic material for microbial growth are probably (1) plant rhizospheres; (2) islands of plant or animal matter which have preserved some degree of structural integrity; and (3) those forces which tend to cause soil aggregate disruption and the exposure of organic materials either to direct microbial attack or to unimpeded diffusion of extracellular enzymes.

(3) The soil microflora is part of the decomposer community and appears to be composed of members of almost every taxonomic group of micro-organisms. Qualitatively, specific microfloras have been correlated with specific soil types only in extreme cases. The total concentration of living microbial tissue in any soil is unknown. In the soil, micro-organisms live in micro-environments whose chemical and physical attributes differ from those measured macroscopically in soil samples. In such micro-environments, cells grow largely in the form of micro-colonies containing some hundreds of cells. When the constraint of this size of colony is applied to the water filled pore-space at 100 cm suction, wet space for microbial proliferation is reduced to the same order of magnitude as that already occupied by micro-organisms in the model soil.

(4) In a soil bearing a climax canopy there is a net zero organic matter accumulation. Microbial mineralisation of organic materials balances annual organic input. Considering a one-year time constant, microbial activity and proliferation is probably limited by available nutrient concentration. A regular annual cycle of microbial growth and activity takes place with peaks in both parameters at the beginning and end of the growing season; the latter peak being the larger. The effect of temperature and rainfall on soil micro-organisms is obscure and probably indirect, reaching them after modification of the soil fabric and canopy activity. Short-term oscillations in the biomass and activity of soil bacteria occur with periods of 35-130 hours in

the model soil. Oscillations (almost certainly of a different origin) with similar periods occur in field soils. In the model soil, the amplitude of the oscillations is convergent; this occurs in real soil, temporarily divergent oscillations also occur. There appears to be an alternation between high amplitude, rapidly decaying oscillations resulting from point stimuli and lower level activity of a more sustained nature arising from extended stimuli. Microbial growth and activity at this scale is likely to be self-limited. It appears unlikely that there is any true short-term steady-state in microbial activity and growth. The composite activity of the soil microflora is likely to be the sum of a series of convergent and temporarily divergent oscillations of varying(decaying) amplitude and period resulting from the growth and activity of organised partly independent units within the community.

E P I L O G U E

The importance of novel techniques and more important, novel approaches to quantitative soil microbiology has been stressed throughout this thesis. Modern apparatus and techniques which could be applied to soil microbiology already exist. The time is appropriate therefore to see the era of the loop, the slide and the petri dish in its proper perspective. Classical techniques have been essential in laying the foundations of the subject, but to avoid stagnation, new approaches must be adopted. Soil microbiology need not be equated with qualitative natural history; it is a subject which can be treated quantitatively and which bristles with problems worthy of the attention of specialists in the various branches of microbiology and its parent sciences.

APPENDIX A

PODZOLS AND PODZOLISATION

Podzols occur under humid conditions and are best developed in cold to temperate regions south of the tundra zones. The dominant factor in podzol development is the prevalence of intense leaching owing to a continued excess of rainfall over evaporation. Podzols may be regarded as completely leached soils, $CaCO_3$ and $CaSO_4$ being present only as fugitive constituents; their reaction is thus acid and although they may develop on any kind of parent material, their degree of development is affected by base status and permeability. Thus their distribution is also markedly affected by geological conditions. Their natural vegetation is generally forest or heath; it is of interest to note that in its meagre demands on the plant nutrient content of soil, heath vegetation exhibits a close resemblance to coniferous forest. Because of their widespread occurrence in Russia and Northern Europe, podzols have been well studied and much detailed information is available on all aspects of this group of soils.

The basic morphology of a podzol profile is illustrated in Figure A(i) (simplified after Robinson, 1949). Because of the basepoverty of these soils, vegetation decomposition is primarily fungal; this results in the accumulation of a raw humus layer which in the almost complete absence of earthworms remains sharply delineated from the lower horizons. In the most extreme cases (humus/iron podzols) transported humic materials and sesquioxides accumulate in the B horizons, the latter occurring at a lower level than the former. In extreme cases this leads to orstein (hard pan) formation at the junction of B_1 and B_2 which

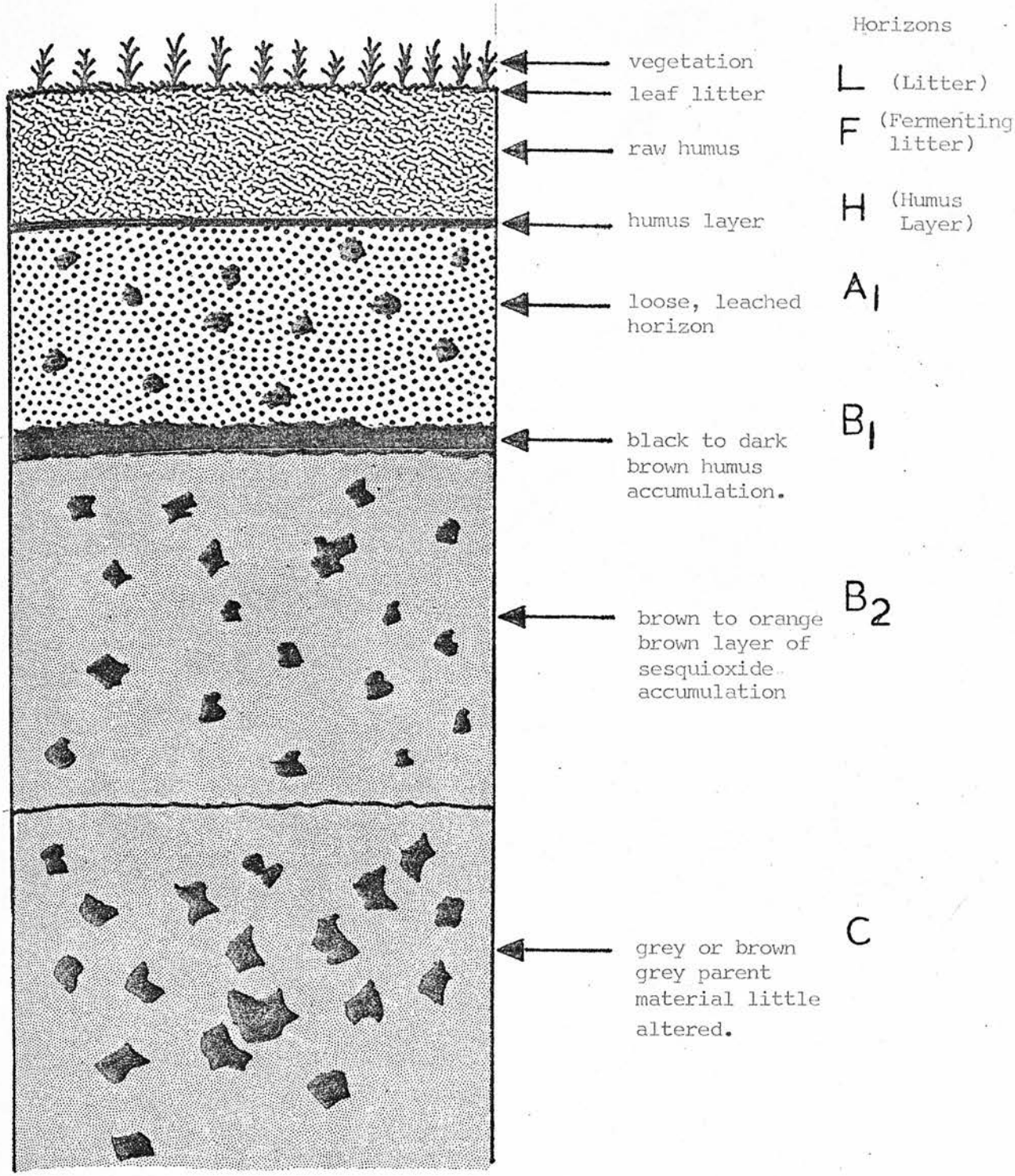


Figure A(i). The morphology of an idealised podzol profile (Simplified after Robinson, 1949).

changes the drainage pattern and aeration status of the profile ultimately leading to the development of a peat and changes in the vegetation.

As mentioned earlier, the podzol chosen for the present study had been examined by the Scottish Soil Survey. The fundamental properties of this soil are summarised in p. 210 - 212 from unpublished data of the soil survey (Ragg, pers. comm.). As well as the abundance of climatic, physical and chemical data on podzols, there exists an extensive literature on their microbiology (e.g. Gray, 1935; Burges, 1963; Williams, 1963; Parkinson and Coups, 1963; Davies and Williams, 1970; Nepomiluev and Kozyrev, 1970).

Description of soil at sampling site

Site:- Glentress Forest, Peebleshire. Nat. Grid NT/283424

Altitude: 535 m slope/aspect 8°/S.S.E.

Association: Ettrick Series: Minchmoor

Soil: Humus/Iron podzol.

Parent material: Coarse, loamy skeletal drift derived from Greywacke and shale.

Vegetation: Calluna vulgaris, Deschampsia flexuosa, Vaccinium myrtillus, V. vitis-idaea.

	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
Rainfall mm	244	216	188	209	216	208	224	232	227	243	235	234
Temperature. °C	0.4	0.1	2.2	4.5	7.5	10.6	11.3	11.2	9.6	7.0	2.2	0.5

(Climatic data = long term averages; rainfall 1960-1972, temperature 1916-1950; extrapolated from Met. Office regional profiles on basis of recordings at Glentress NT/285397).

Chemical Analysis:

Horizon	depth (cm)	% loss on ignition	% sand	% silt	% clay	Exchangeable cations m.eq/100 g Ca	Mg	Na	K	H	% base saturation	pH, H_2O	pH, $CaCl_2$	Organic fraction % C	% N	C/N	%OM	P.mg P_2O_5 per 100g
H	4-0	68.3				5.83	2.00	0.39	0.78	67	76	11.8	3.6	2.9	39.6	1.45	27.3	68.2	285
A_1	0-8	17.7	56	12	9	1.08	0.39	0.07	0.23	25.7	27.4	6.4	3.8	3.2	9.39	0.51	18.4	16.1	176
B_1	8-13	25.9				0.96	0.16	0.04	0.2	51.2	52.6	2.6	4.0	3.6	11.55	0.6	19.2	19.9	285
B_2	13-30	11.1	56	13	8	0.93	0.03	0.03	<0.02	17	18	5.7	4.2	4.0	2.85	0.14	20.4	5.0	234
Cx	30-40	7.9	25	11	4	0.92	<0.02	<0.03	<0.02	25.9	26.9	14.9	4.3	4.2	0.79	0.06	13.2	1.4	144

Profile description:

L	7 - 5 cm	*Calluna* litter
F	5 - 4 cm	decomposing litter
H	4 - 0 cm	black raw humus + mineral material
A_1	0 - 8 cm	dark grey and dark reddish brown fine sandy loam; common subangular subrounded stones; abundant roots; sharp boundary.
B_1	8 - 13 cm	reddish black fine sandy loam; abundant subangular stones; abundant roots; sharp boundary.
B_2	13 - 30 cm	yellowish-brown gritty sandy loam; abundant angular and subangular stones; very friable; common roots; merging boundary.
Cx	30 - 40 cm	yellow-brown fine loamy sand; abundant angular stones; very firm, compacted; roots rare; sharp boundary.
II C	40+ cm	yellow-brown greywacke rubble with gritty loamy sand interstitial material.

APPENDIX B

COLLECTION AND ANALYSIS OF CO_2

(1) Collection of CO_2

The air-supply system and CO_2-scrubbing pre-treatment presented some difficulties which were eventually resolved. The general scheme adopted is indicated in Figure B(i). A variety of sources of compressed air were tried, a cylinder was satisfactory but too short-lived while the compromise between noisiness and power in compressors was difficult to achieve. Ultimately a motor-driven diaphragm pump incorporating an air-receiver and pressure-guage provided suitable performance. A rotameter calibrated between 100 and 1000 ml/min was used to measure and control air flow-rate.

In view of the expected small amount of CO_2 from the soil samples, it was desirable that the background CO_2 (in the flushing air) be as low as possible. It was found that Dreschel bottles with open-ended inlet tubes did not remove a significant proportion of atmospheric CO_2 when filled with 150 ml M NaOH and aerated at 300 ml/min. (CO_2 was determined chromatographically p.223). Accordingly, two Dreschel bottles were used in series, each fitted with a sintered glass sparger. Hyamine hydroxide solution was found to be ten times as effective an absorbant as NaOH at the same molarity. Its major disadvantage of excessive frothing however ruled out its routine use.

10M NaOH in two series Dreschel bottles fitted with sintered glass spargers proved satisfactory for periods of up to 24 hours only. After this time, the pores in the sparger in the first bottle in line became blocked, cutting down gas flow and bubble size; this resulted in foaming over into the second bottle. This was presumed

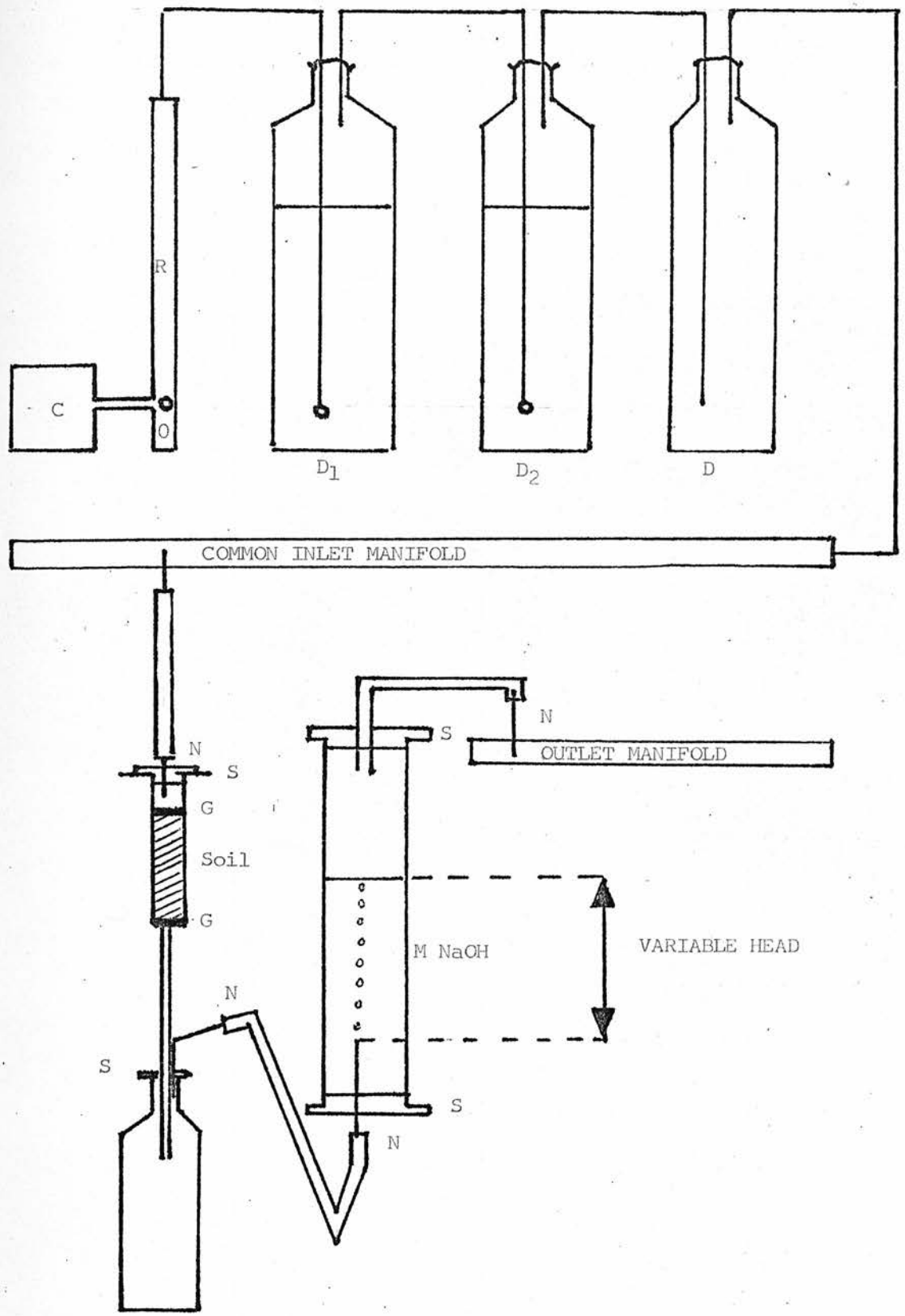


Figure B(i). Air supply, scrubbing and CO_2 trapping apparatus.

[C = air supply; R = rotameter; D = Dreschel bottle, 1 and 2 with 10M NaOH; 21G x 1½ needle; S = 'Suba-Seal' closure; G = glass wool.]

to be caused by Na_2CO_3 precipitation in the sparger (the salt is insoluble in strong alkali). The problem was overcome by using Keiselguhr aquarium spargers. These appeared to dissolve very slowly in the alkali, the net effect being that the pores did not become seriously blocked until the sparger had been in use for ca. 1 week. This procedure was successful in reducing background CO_2 to an acceptable level. Compressed air from the alkali blow-over trap (Figure B(i)) was led into a sealed length of rubber compression tubing. This acted as a common manifold for all replicate soil samples. Pressure was bled from it via 21 G x 1½" syringe needles into 3/16" i.d. rubber tubes connected via another needle to the soil columns through No.36 "Suba-Seal" flat closures in the necks of the disposable syringe barrels. Effluent air passed out of the syringes via plastic pipes into bottles sealed with another rubber closure as above. (This bottle was primarily a receiver for effluent liquid; see p.169 et.seq.). Pressure was bled from this seal via a needle into the CO_2 traps described below.

CO_2- trapping was effected by bubbling the effluent air through M NaOH. Inlet tube orifice area and alkali head were found to be critical in determining the minimum back-pressure (hence flow-rate) at which all the ($\hat{=}$ 30) traps passed air. For these reasons, inlet tubes dipping into the alkali had to be discarded. The problem was largely overcome by introducing the gas to the trap from the bottom via a needle through a rubber seal (Figure B(i). This system had the advantage that small bubbles (hence high surface area/volume ratio) of relatively constant size were produced in all replicate traps. Equally important was the fact

that the effective head of alkali was simply adjustable by sliding the needle in the seal, thus allowing individual adjustment of flow-rates in all traps while retaining the same volume (10 ml) of alkali in each trap.

Effluent gas from the traps was led via a needle into a common rubber outlet manifold. This served the purpose of providing a CO_2-free atmosphere at positive pressure for all traps, hence diminishing problems of CO_2 back-diffusion in the event of individual trap failures. A soap-bubble flow-guage was attached to the outlet manifold so that large leaks in the system could be detected by its comparison with the rotameter reading. Small leaks were searched for with soap solution. It was found necessary to discard all rubber components which had been pierced by needles after one experimental run since without the needles in position, they leaked under positive pressure. Needles of smaller diameter than 21 G were found to become blocked when pushed through thick rubber; hence only 21 G needles were used throughout.

(2) Titrimetric analysis of trapped CO_2

Routinely, the difference in CO_2 production between triplicate soil columns per treatment and triplicate empty columns was determined. This procedure eliminated the effect of residual CO_2 in the flushing air and CO_2 already dissolved in the alkali. A number of methods of analysis were tried until a satisfactory one was found.

(a) Titration of residual alkali

Initially CO_3^{--} was precipitated by the addition of a known

volume of M $BaCl_2$. The suspension was filtered and an aliquot of the filtrate titrated against M HCl to a bromothymol blue end point. The difference in alkali molarity between the control and the test traps allowed the amount of CO_2 absorbed to be calculated. The process was not satisfactory for a number of reasons including the facts that:-

(1) The precipitation and filtration stage was tedious and difficult to carry out since the precipitate formed slowly and was of small particle size; and

(2) The resulting CO_2 concentrations were related to a small difference between two large figures (the alkali normalities) hence precision was low.

(b) Titration of CO_3^{--} in the presence of trapping alkali. Duplicate or triplicate 2 ml samples were removed from each trap and titrated with N/5 HCl to a phenolphthalein end-point; (Saturated phenolphthalein phosphate in 3:2,ethanol:water), saturated aqueous methyl orange was added and the titration was continued to a red end-point. During the first titration, the reactions

$$Na\ OH + HCl \rightarrow NaCl + H_2O$$

$$Na_2CO_3 + HCl \rightarrow Na\ HCO_3 + NaCl$$

occurred. During the second titration, the reaction

$$Na\ HCO_3 + HCl \rightarrow CO_2 + H_2O + NaCl$$

took place. Hence the difference between the end points is equivalent to half the carbonate present and the trapped CO_2 concentration may be simply estimated.

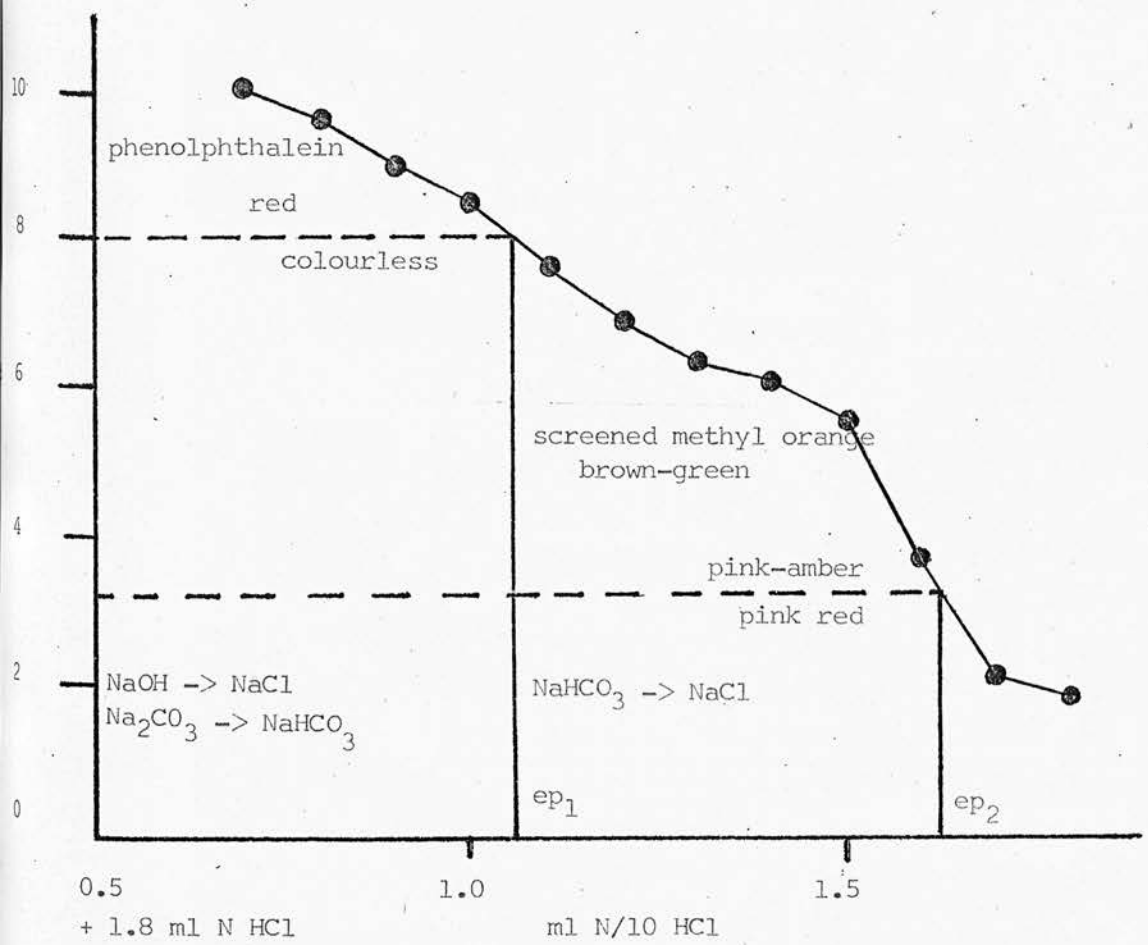


Figure 8(ii). Course of carbonate titration in the presence of residual alkali.

A practical problem in this method is that phenolphthalein (red) begins to fade when only 80% of the CO_3^{--} has been converted to HCO_3^- and the decolourisation is not complete until 3-5% of the acid is in excess (Belcher and Nutten, 1960). This problem can be overcome by comparing the titration to a standard HCO_3^-/phenolphthalein colour mixture. In this case however, where results are calculated as a difference from controls, its relevance is different in that making the phenolphthalein and methyl orange end-points closer together, it decreases the precision of the method slightly. Another problem is the poor colour characteristics of methyl orange. The change from orange to red is diffuse and difficult to detect. It was found that this end-point was more clearly ascertained if screened methyl orange (B.D.H. Indicator 3046) or bromophenol blue was used. Considerable between-batch variation in the '3046' indicator was experienced, some batches being little better than methyl orange.

This method was refined and used routinely on duplicate samples per trap. The 3 ml samples were taken close to the phenolphthalein end-point by the addition of a predetermined amount (ca. 1.85 ml) N HCl from a stock burette. Titration to the first end-point (Figure B(ii)) was carried out carefully with M/30 HCl and the difference between the two end-points determined in terms of this concentration of acid. The pH curve of the reactions involved, is shown in Figure B(ii) (data taken from the titration of a trap during the course of an experiment). Since large numbers of titrations were being carried out routinely, some streamlining and automation of the system was obviously necessary. Accordingly, a Conway-type semi-automatic micro-

burette was constructed. This system proved to be most satisfactory in routine use, being simple to operate reproducibly.

APPENDIX C

DESIGN AND CONSTRUCTION OF THE PEDOSTAT

(1) Theory

The results of the point-stimulus model soil experiment indicated that continuous measurement of CO_2 production from incubated soil samples was likely to be just as valid as biomass measurement in the study of microbial community dynamics. It was also experimentally more advantageous than destructive analyses depending on parallel soil cultures. In logical progression from the point stimulus study, continuous stimulation was considered. In nature, a wide variety of stimuli is likely to be experienced by the soil microflora. These may include rainfall, temperature changes or soil movement as a result of physical or biological factors. All such stimuli are likely to change the availability of substrates and alter the micro-environments to favour specific groups of micro-organisms or micro-organisms in general. The net result of such influences is likely to be that microbial proliferation takes place in bursts resulting from sporadic stimuli while growth is sustained by longer term stimuli. This combination of "batch" and "continuous" growth is likely to be manifested as the modification of whichever basic growth pattern is present by the more over-riding effects of the alternative type of stimulus.

The study of a soil which has received a point stimulus is one of an activated but degenerating system. Being effectively a closed system, it can do little but decline and there is no stable equilibrium except when all the substrates are catabolised and all the cells are dead. An open system continuously or sporadically stimulated is an attractive model to study. Provided the response of the microflora is some function of the growth promoting stimulus, there is the possibility of establishing an equilibrium

in activity. The open system allows for the addition of nutrients and so such a model is not necessarily of limited time-span. The nature of the growth-sustaining stimulus is important since in a study of a mixed microflora it should be relatively non-specific in its effects. For this reason, the nutrient-leaching effect of percolation with water was considered a suitable stimulus. Such a system could be used to mimic rainfall, a natural long and short-term stimulus. It also allows for the manipulation and maintenance of the microflora by the addition of specific dissolved nutrients.

Because of the limitations of the various methods of community analysis, it is useful to be able to measure as many growth-correlated parameters as possible. In view of the heterogeneous nature of soil and soil microfloras, and the destructive nature of enumerative procedures, provision must be made for satisfactory replication in any experimental system designed to study continuously stimulated soil. Accordingly, a piece of apparatus was constructed which was sufficiently versatile to allow these ends to be met. Another important design feature - that the apparatus should be capable of development and modification for future studies was also borne in mind.

The importance of continuous culture experiments in microbial ecology has been acknowledged from time to time in the literature (e.g. Jannasch, 1965). Many soil percolating devices have been constructed (e.g. Lees, 1949; Greenwood and Lees, 1956; 1959; Sharp and Taylor, 1969); most of these have been designed for biochemical studies and are not directly suitable for the type of

study envisaged. Open-system continuous flow apparatus has been constructed and used by Macura (1960; 1961; 1964; Macura and Malek, 1958; Macura and Kunc, 1961). Again the system was devised for biochemical studies on a small number of replicate soil samples and for this reason was not applicable to the present study.

The first major problem in the design of a continuous culture machine for the study of soil microfloras (termed 'pedostat' in this study) is that of pumping liquid medium to a large number of replicate soil cultures at identical flow-rates. The problem was compounded by the fact that ideally, separate reservoirs were required for each soil culture and a wide range of flow rates was also desirable. The only commercially available pumping systems capable of answering such requirements are multi-channel variable speed peristaltic pumps. These were not completely satisfactory for a number of reasons, including cost, inter-changeability of culture unit components, wearing of pumping tubes, abundance of tubing couplings etc. Accordingly, a simpler system was designed and constructed.

The principle of the pedostat is illustrated in Figure C(i) Soil incubation containers, CO_2 trapping and aeration arrangements have already been described (p. 213). Individual eluent reservoirs for each soil culture took the form of 50 ml plastic disposable syringes. Liquid was expelled from these reservoirs through 21 G needles into the soil columns by pressure from the movable plate A. In practice, plates A, B and C were circular and accommodated 30 reservoir/ soil column units around

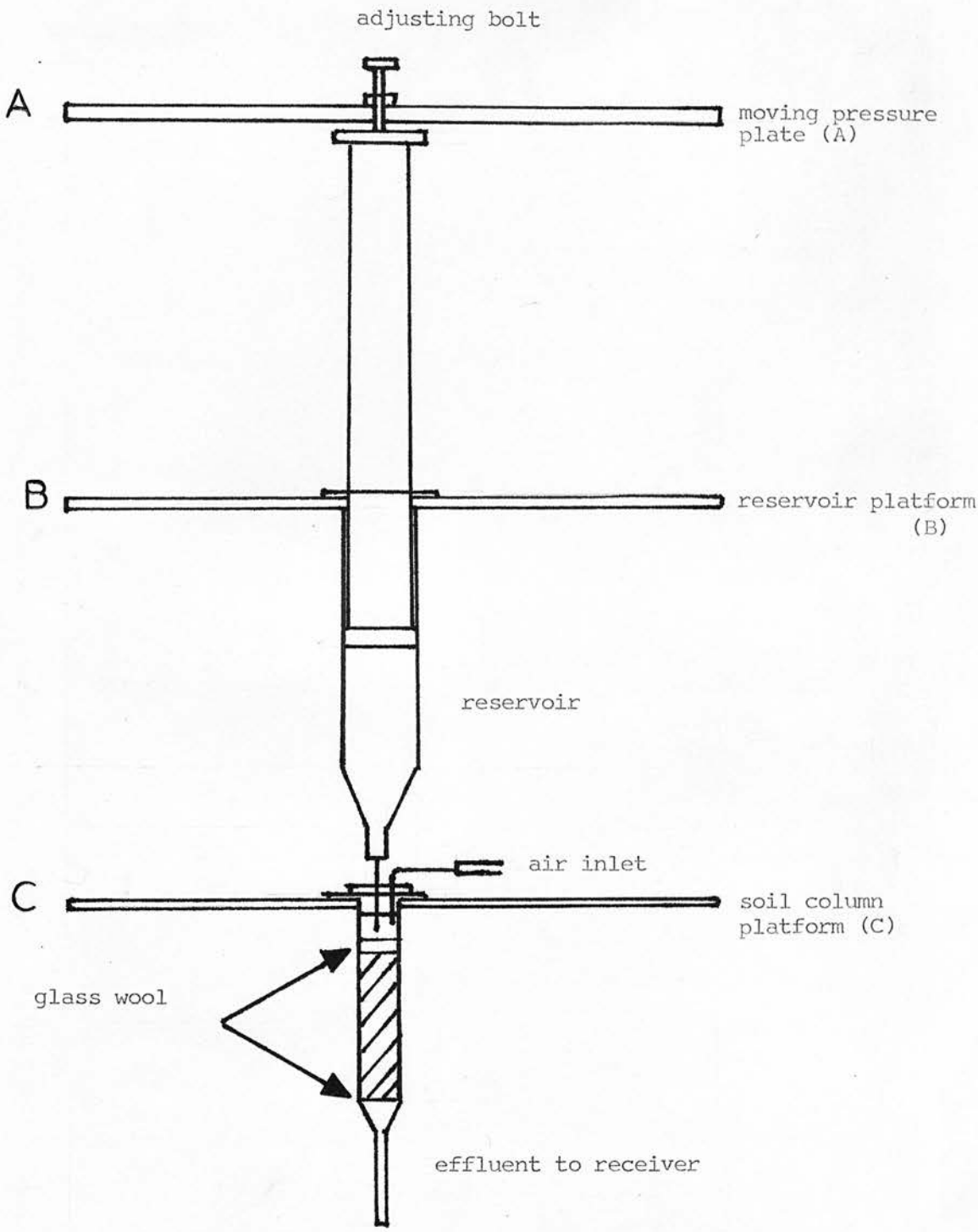


Figure C(i). Pedostat liquid pumping system. 30 replicates of the above system were arranged around the circumferences of plates (A), (B) and (C).

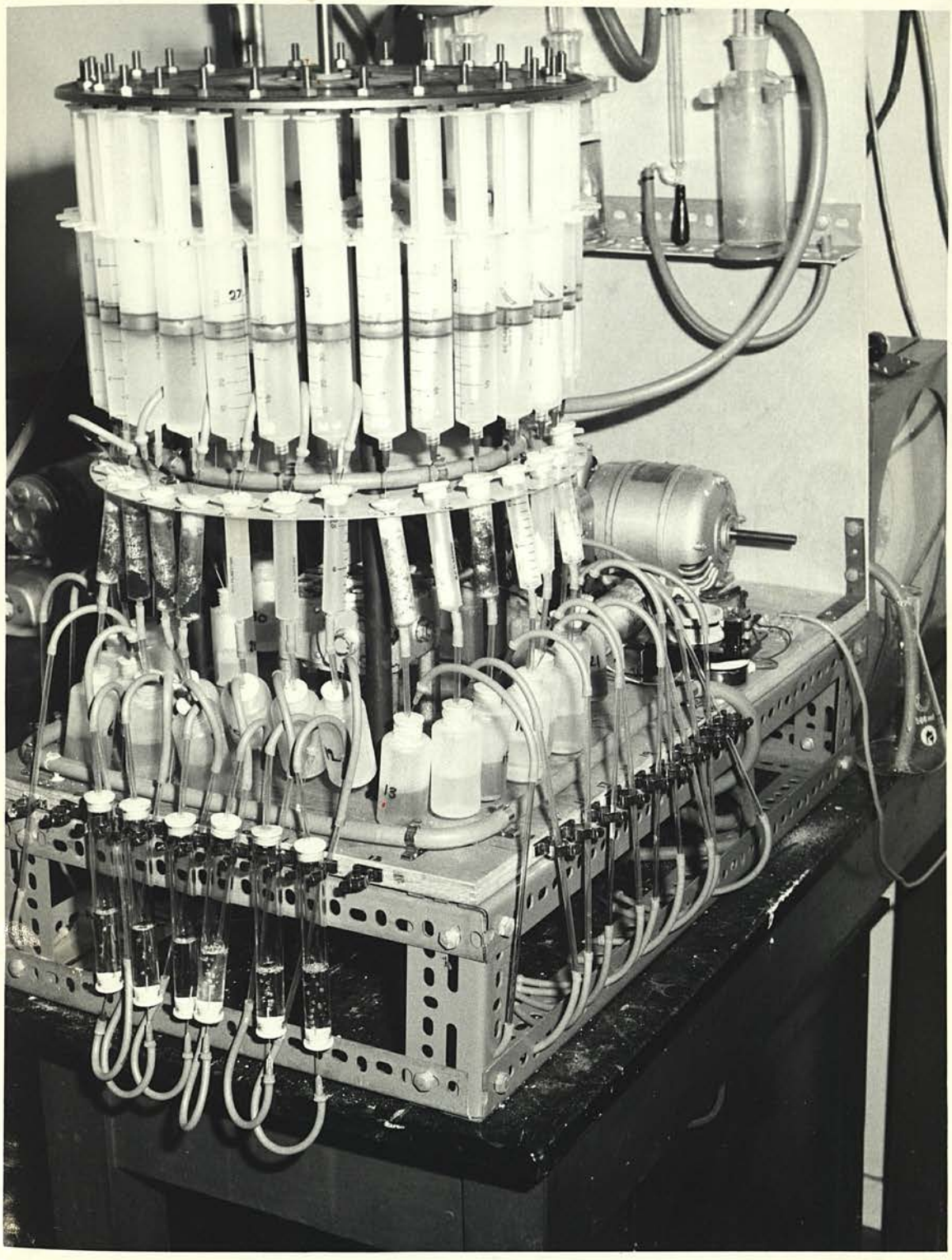


PLATE 10 The pedostat.

their circumferences as illustrated in Plate 10.

(2) Construction details

The pressure plate (A) was drawn down by a threaded rod (½" diameter, 26 threads/in) against a fixed nut by a worm drive 50:1 reduction gearbox (David Brown, RADICO). The worm drive was driven through a variable speed reduction gearbox (Zero-Max) by an electric motor (Parvalux). The electric motor was connected in series to a programmable timer (Impex). Thus by adjustment of the variable speed gearbox (arbitrarily calibrated) and selection of switching programmes a wide variety of flow-rates could be obtained. The timer allowed the machine to be turned on or off for any integral period of minutes, the maximum period between successive switchings being 60 minutes. Practically, this allowed flow-rates between .0003 and 1.28 ml/min (p. 225) to be obtained. The method by which the final drive was effected was illustrated in Figure c(ii).

The drive-spindle was connected to the gearbox through a clutch which could be disengaged when it was required to rack the pressure plate manually. The basic components of the final drive were the inner (I) and outer (O) concentric tubes and the drive-spindle (D). A threaded nut was fixed in the bottom of I and rotation of D against this caused I to rise relative to O. Rotation of I was prevented by the engagement of a tooth on the inner surface of O in a longitudinal slot in I. The top of I was fixed to the reservoir platform (B). The two static plates (B) and (C) were bolted firmly against a rigid baseboard by threaded rods and tubular spacers.

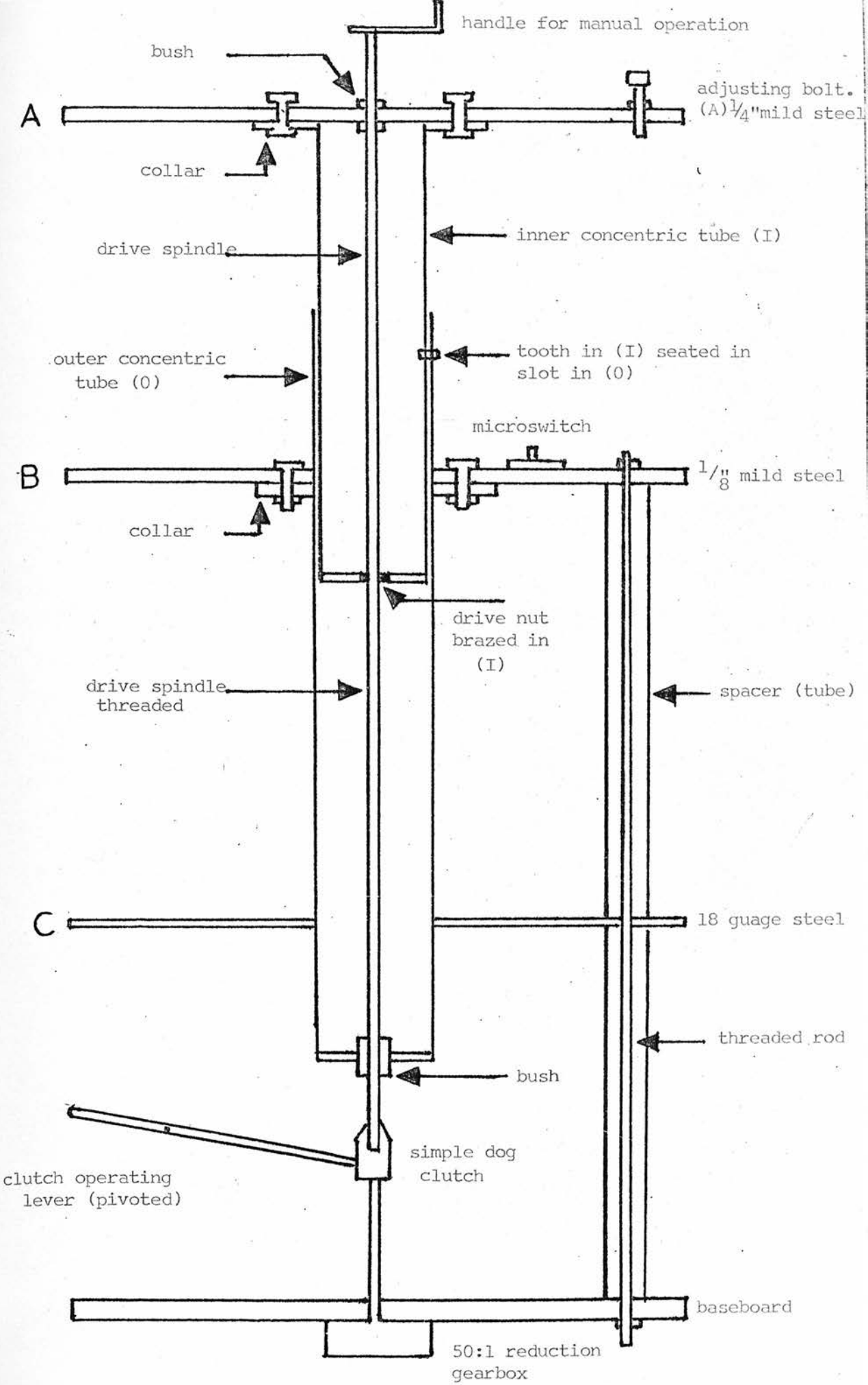


Figure C(ii). Construction of the pedostat.

Adjusting bolts were included in A to ensure that all reservoirs started to deliver at the same time when the pressure-plate was depressed. Coupled with the manual operation facility, this allowed any reservoir to be exchanged rapidly during the course of an experiment; all reservoirs could be simply refilled and replaced so that experiments could be continued indefinitely. Although aseptic running of the machine was not considered in this study, it was comparatively simple to achieve with the system employed.

(3) CO_2 analysis

In use, the pedostat provided a large number of alkali samples for analysis. The titrimetric method described earlier (p. C(iv)) was simply not fast enough to accommodate samples at the rate at which the pedostat produced them. Accordingly, a method based on CO_2 analysis by a katharometer was evolved. The basic principle of this approach was to release CO_2 from a sample of alkali in a sealed bottle and by means of a gas sampling valve (described below) to introduce a known volume of gas into the carrier-stream of a gas chromatograph equipped with a katharometer detector and recorder. A Pye 104 gas chromatograph fitted with a 9 ft 80-100 mesh silica-gel filled glass column was used in this study. The carrier gas was O_2-free N_2 at *ca.* 26 ml/min; the operating temperature was 140°C giving a CO_2 retention time of *ca.* 4½ minutes under the conditions described.

Sample preparation involved pipetting 3 ml trapping alkali into a $^1/_4$ oz glass vial the volume of which had been previously determined. This was sealed with a 'Suba-Seal' flat rubber closure and 1 ml of

air replaced by concentrated HCl using a syringe. This liberated all CO_2 from solution. On injection into the chromatograph, the CO_2 peak was preceded by an oxygen peak and a variable hydrogen peak resulting from reaction between the acid and the syringe needle. Sampling was effected by means of a standard volume gas sampling loop (1 or 0.5 ml) (Pye accessory No. 792082) The inlet port of the loop was equipped with a 21 G needle and an excess of gas (2 ml) was displaced from the $^1/_4$ oz vial through this needle by the injection of dilute HCl through the rubber closure.

This sampling arrangement coupled with suitable equilibration delays while the loop was being filled and flushed allowed the sample volume to be selected at very close to atmospheric pressure. This obviated any dilution of the sample which might be caused by drawing air into a syringe filled at negative pressure and premature expulsion of part of the sample from a syringe filled at positive pressure. The slight difference in gas sample density in the sealed vial and in the loop open to atmospheric pressure produced an undetectable difference in measured CO_2 concentration, peak heights being reproducible to 99% from the same sample vial. The relationship between CO_2 peak height and CO_2 concentration in the trapping alkali was determined by the use of gas mixtures of known concentration prepared in a sealed flask and injected as described above. As expected, the function was not linear but took the form of a shallow curve the precise shape of which had to be determined each time the chromatograph was used to analyse a series of (usually 120) samples.

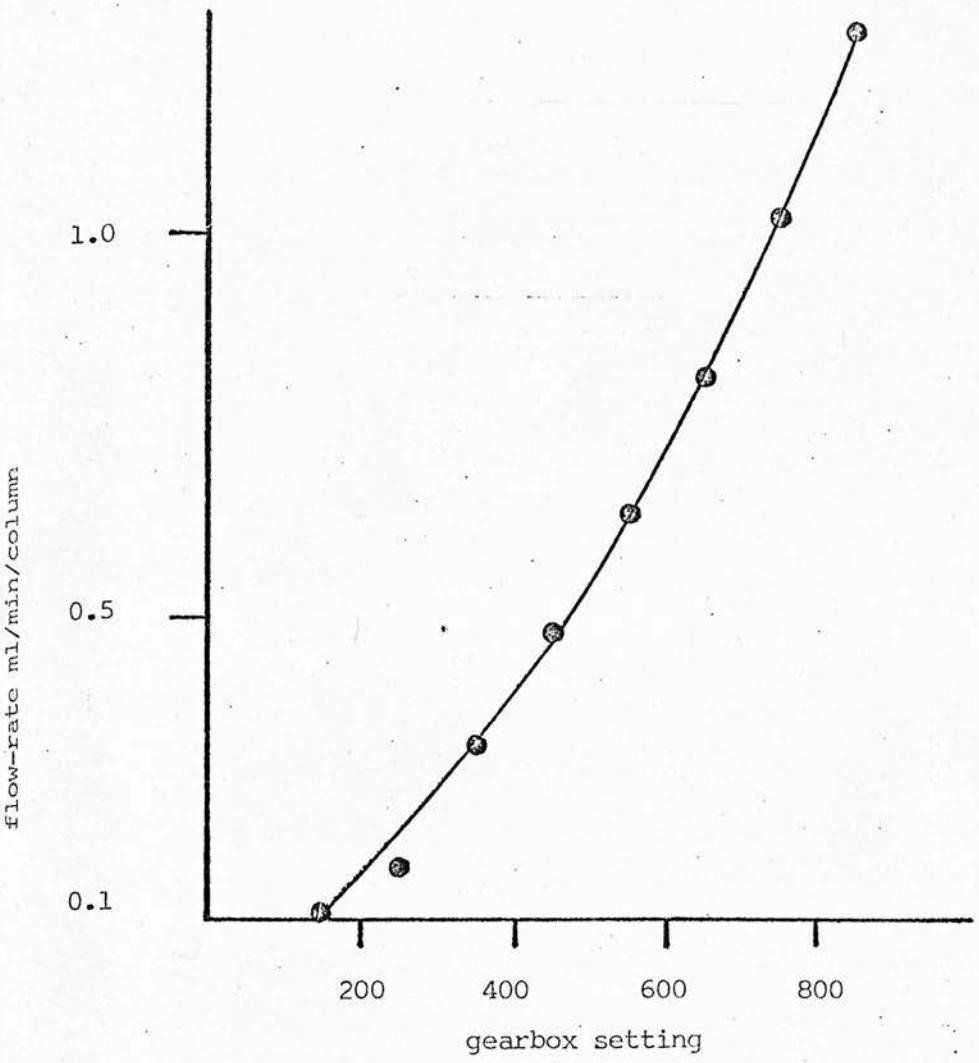


Figure C(iii). Gearbox speed/flowrate calibration.

(4) Flow-rate calibration

Ten of the syringes destined to carry soil in the pedostat were removed at random and their outlets sealed with wax. These were weighed and equipped with extra syringe needles to allow air displacement. The reservoirs were filled with water and timed runs made at various settings of the variable speed gearbox. The gearbox speed settings were in the form of arbitrary units on a useable scale of between 100 and 850. The weight of water delivered in a five minute run at a particular speed plus the water delivered as a result of pressure equilibration in the consecutive five minute period was used to construct a flow-rate calibration of the gearbox. This is shown in Figure C (iii). Maximal between-sample variation of 1.1-3.5% in flow-rate was noted over the range of speeds tested. It can be seen that flow-rate is a simple function of gearbox setting and with the aid of the calibration, any desired rate in the available range could be easily selected. The range was considerably extended by the use of the timer.

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